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DNA binding properties of histone-like protein HU from Deinoccus radiodurans suggest involvement in DNA recombination

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DNA BINDING PROPERTIES OF HISTONE-LIKE PROTEIN HU FROM *DEINOCOCCUS RADIODURANS* SUGGEST INVOLVEMENT IN DNA RECOMBINATION

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TABLE OF CONTENTS

LIST OF FIGURES

Page

ABSTRACT

The Histone-like protein HU is ubiquitous in eubacteria. Usually with a length of ~90 amino acids, they are predominantly homodimeric, with sequence and structural homology. *Escherichia coli* HU is involved in DNA repair and recombination. The crystal structure of *Anabaena* HU shows that it binds DNA with prolines intercalating into the DNA backbone, introducing two kinks at a spacing of 9 bp and bending the DNA through a variable angle of 105-140°. *Deinococcus radiodurans* is a gram positive mesophile, capable of reconstituting its genome from 1000-2000 double strand breaks incurred due to exposure to environmental extremes.

In the first study, *D. radiodurans* HU (DrHU) is characterized in terms of its DNA binding properties. The binding site size of DrHU is the largest so far reported, ~50 bp. DrHU binds preferentially to four-way junction DNA with half-maximal saturation of 18 ± 2 nM. In distinct contrast to *E. coli* HU, DrHU has no marked preference for DNA with nicks or gaps compared to perfect duplex DNA, nor is it able of mediating circularization of linear duplex DNA.

In the second study, the N-terminus of DrHU was truncated, generating ΔDrHU, and the functional role of the N-terminus investigated. ΔDrHU exhibits a binding site size of 17 ± 1 bp similar to HU homologs from other mesophiles. ΔDrHU also binds preferentially to four-way junction DNA, but protects the crossover rather than the junction arms protected by DrHU. The melting temperature of ΔDrHU of 46.4 ± 0.1°C is similar to that of HU from mesophiles. DrHU interacts with other *D. radiodurans* proteins(s) in the presence of four-way junction DNA, suggesting its role in DNA recombination.

In a similar study with the HU homolog from *Helicobacter pylori* (HpyHU), the protein binds stably to four-way junction DNA with half-maximal saturation of 5.0 ± 0.5 nM. Thermal denaturation of HpyHU measured by circular dichroism spectroscopy yields a Tm = 56.4 ± 0.1˚C suggesting greater than average thermal stability. Mutagenesis of HpyHU suggests that a differential target site selection of HU proteins is achieved through their individual capacity for inducing the required DNA bend.

CHAPTER 1

INTRODUCTION

In eukaryotes, the genome is organized into chromatin, a structure that is differentially accessible for replication and transcription [1]. The fundamental block in eukaryotic chromatin is the nucleosome, a bead-like structure composed of an octamer of histone protein subunits with the DNA wrapped around its surface [2]. Additionally, in eukaryotic cells the nucleosomes are deposited non-randomly on the genomic DNA, making it differentially accessible for developmental processes [3]. As compaction and accessibility of genetic material are likely important for life, it was postulated that a similar system may be operating in bacteria. Bacterial proteins, termed histone-like, are abundant non-specific DNA binders and their primary function is to organize the genomic DNA [4]. There are several members of this class in prokaryotes – primarily, H-NS, Fis, the HU homolog Integration Host Factor (IHF), LRP, and HU.

Short Account of the Members of the Histone-like Family

H-NS (histone-like nucleoid structuring protein), or H1 or B1, as it was initially named, is an abundant, heat-stable, DNA-binding protein found in bacteria [5, 6]. With a molecular mass of 15.6 kDa, it is neutral, migrating with a p*I* of about 7.5 [6, 7]. It contains a large number of charged residues, which unlike the eukaryotic histones or other DNA-binding proteins are acidic and not basic. It does not contain a typical DNA-binding motif (*i.e*. helix-turn-helix or zinc finger), but has a stretch of positive charges at the C-terminal end which may be involved in DNA binding. It forms a dimer in solution, with small amounts of trimer and tetramer [8, 9]. It has three isoforms, differing in isoelectric point, but present in equimolar amounts [7]. H-NS is induced by cold shock and its concentration increases three to four fold in comparison to its level under normal growth conditions, thus repressing transcription of a number of genes [4, 10]. It

plays an antagonistic role with HU, affecting chromosome replication and partitioning [10]. It is abundant (22,000 molecules per cell or 1 H-NS dimer per 440 bp DNA, [11]) and autoregulates its own expression in the cell. H-NS binds DNA non-specifically, very strongly [12], and prefers curved DNA and bends non-curved DNA [13], inducing topological changes, thus indicating that it compacts the genome [7, 14].

FIS (factor for inversion stimulation) is a 11.2 kDa, heat-stable, DNA-binding protein in *Escherichia coli*. It is a pleiotrophic transcriptional regulator altering the pattern of gene expression through direct control at transcription initiation or indirectly of many genes [15-17], including activation of rRNA synthesis [18]. It is the most abundant protein in growing cells (60,000 molecules per cell, [11]) and modulates the activity of gyrase and homeostatic control of DNA supercoiling [19]. FIS is able to stimulate site-specific DNA inversion, chromosomal replication, phage integration/excision, DNA transposition and illegitimate recombination reactions by binding to an enhancer sequence and bending the DNA [20-26].

Several site-specific recombination systems in *E. coli* are stimulated by a host factor called IHF [27], which has more than 45 percent identical or similar residues with HU [28]. It is a basic protein composed of two non-identical subunits, IHF-α and IHF-β, having molecular weight 11,224 and 10,581, respectively, encoded by *E. coli* genes *him*A and *hip*, respectively [27]. There are about 3,500 molecules (dimers) per cell, yielding an intracellular concentration of about 6 μM [28]. IHF binds DNA, and three sites 30-40 bp long with conserved recognition sequences (YAANNNNTTGATW, [29, 30]) have been detected on *attP*-containing DNA [31]. IHF recognition sites are also found in the *att* region of bacteriophages φ80 and P22 [29], the terminal region of insertion element IS1 [30], in phage 21 *cos* site [32], and upstream of translation initiation codons or close to promoters of several genes [31, 33, 34].

Lrp protein (leucine-responsive regulatory protein) is a 15 kDa, DNA-binding protein that works as a global transcriptional regulator affecting the transcription of at least one-tenth of all *E. coli* genes, most of which are expressed in stationary phase [35]. It can exist as dimers, tetramers, octamers and hexadecamers with a moderate high copy number (approximately 3000 dimers per cell) [36, 37]. The crystal structure of LrpA from *Pyrococcus furiosus* revealed the protein has a N-terminal domain which has a typical helix-turn-helix fold that binds DNA and a C-terminal domain with an αβ-sandwich fold that functions as a regulatory domain modulating the catabolism and anabolism of many amino acids as well as pili synthesis [38, 39]. Binding to DNA is non-specific and usually co-operative which suggests that the protein acts less-specifically as a DNA-organizing protein, contributing to the packaging of the chromosome [37, 40], and without any influence on initiation of DNA replication [41]. In *E. coli*, Lrp often acts in concert with other global regulators like CRP (cAMP receptor protein), IHF and H-NS [42-44].

Abundance of HU

HU is a basic, DNA-binding protein capable of wrapping DNA. HU was first isolated from *Escherichia coli* strain U93 (ribonuclease negative), and was called factor U. The letter H was added when there was growing evidence of its similarity to eukaryotic histones [45]. Its primary structure is highly conserved among prokaryotes, and it is found in almost all eubacteria, a few archaebacteria, blue-green algae and also plant chloroplasts, bacteriophages [46] and animal viruses [47]. Recently, histone-like proteins have been reported in the dinoflagellate *Crypthecodium cohnii* [48] with similarities to both eubacterial histone-like proteins and eukaryotic histone H1, thus postulated as an intermediate in the evolutionary scale.

HU proteins are small, usually with length varying from 90-99 amino acids with a molecular weight of ~10,000 daltons. They exist as dimers in solution, which can be heterodimer

as seen in enterobacteria [49], or homodimer as found in other eubacteria [4]. Based on the values of the protein content and the volume of the cell [50], the cellular concentration of HU in the cell has been calculated at an average value of 2.5 ng per μg of cell protein [51], which is about 12,000 molecules (dimers) per cell, which corresponds to an intracellular concentration of about 20 μM. Others have shown that the HU level (about 50,000 molecules per cell or 1 HU dimer per 190 bp DNA) decreases in the stationary phase to less than one-third (15,000 molecules per cell), which is close to the level of H-NS [11].

Structure of HU and DNA Binding

The molecular structure of HU and its interaction with DNA is based on the NMR and X-ray crystallographic analysis of the HU homolog IHF from *E. coli* [52] and the homodimeric HU protein from *Bacillus stearothermophilus*, *Thermotoga maritima* and *Anabaena* [53-58]. The amino-terminal half has two α helices connected by a turn; the carboxy terminal half also forms an α helix. The remainder of the protein has a three-stranded β sheet structure which includes a β ribbon extension in the middle. Two monomers come together to form a compact α-helical "body" capped by β-sheets that extend as two β-ribbon "arms" which are disordered [53, 55]. X-ray and NMR studies have shown that the arms are flexible in solution and their tips are folded [56, 59-61]. *E. coli* IHF and *Anabaena* HU have been co-crystallized with DNA [58] and the structures reveal that the β-ribbon arms lie in the minor groove of the DNA and a highly conserved proline residue at position 63 at the tip of each arm introduces kinks into the DNA at a spacing of 9 bp and causes stabilization of the bending by intercalating into the base-pair stack (Figure 1.1). The DNA can also interact with the positively charged residues on each side of the protein which is variable. Despite sequence and structural similarity, IHF and HU distinguish different DNA substrates. While IHF binds tightly (2-20nM) [62-64] to cognate sites represented

by the consensus WATCARXXXXTTR (W is a A or T; X is a A, T, C, or G; R is A or G), HU does not have any topology-independent DNA-binding sequence. It has low nanomolar affinities for DNA with nicks, gaps, cruciforms, phased loops and with single base insertions 9 bp apart [65-70].

Fluorescence resonance energy transfer (FRET) experiments of IHF-DNA [71] confirmed previous affinity experiments and crystallography results showing that the bending in solution is ~160˚ which also agrees with the DNA cyclization experiments [72]. Whereas IHF binds without cooperativity to 35 bp DNA under different conditions, published results on HU vary with little or high cooperativity of DNA binding with sites varying from 9 to ~ 50 bp [73, 74]. Thus, the short, non-specific 9 bp binding sites are due to the DNA being bound only by the β-ribbon arms, whereas longer binding sites are due to contacts between the more tightly bent DNA and the sides of the protein [for review, 75]. Recent co-crystal structures of HU with DNA reveal that although IHF and HU are conserved, HU is capable of inducing/stabilizing different bend angles of 105-140˚ as observed in the three independent *Anabaena* HU homodimers [58]. The variable bend angle correlates with a more fluid structure of the prokaryotic chromatin compared to that of eukaryotes. This facilitates HU in formation of higher-order protein-DNA complexes that may require different, system-specific DNA bend angles [75]. Molecular dynamics/potential of mean force stimulations show that the large strain generated with the disruption of the base stacking at the kinks in IHF-DNA and HU-DNA complexes, with ~14.1 kcal/mol for the kink in IHF [76], is coupled with the disruption of a large number of surface salt bridges [77, 78]. A recently proposed binding model based on isothermal titration calorimetry (ITC) shows that the binding is enthalpically driven with limited salt dependence. This type of surface salt bridge distribution also plays a role in HU-DNA complexes where the variations in

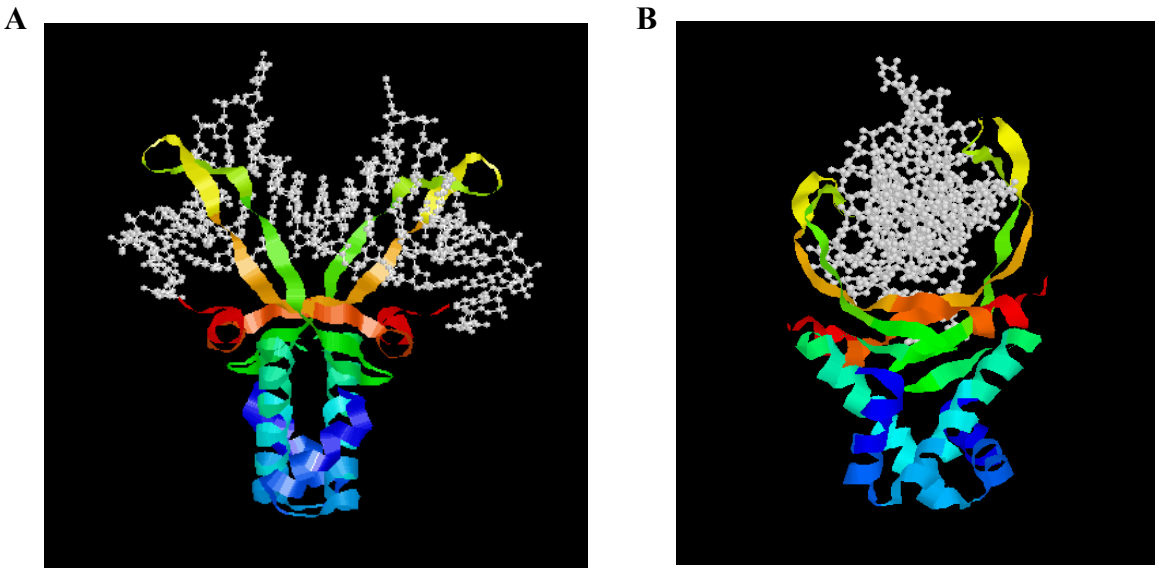

Figure **1.1**. Cocrystal structure of *Anabaena* HU with DNA. (Protein data bank (PDB) code IP 51) [58]. A is turned 90° to get the alternate view B. DNA is shown in ball and stick model and protein in ribbon form.

the binding site lengths are due to the presence/absence of amino acids involved in the formation of the salt bridges distal to the site of kinking [79, 80]. In HU homologs, Lys3 is proposed to form a salt bridge with Asp26 which results in shorter binding sites, whereas in Transcription Factor 1 (TF1, HU homolog from *Bacillus subtilis* bacteriophage SPO1) Asp26 is absent, thus Lys3 contacts DNA 8-9 bp away from the kink, forming a 37 bp binding site [80].

In *E. coli* where most of the studies on HU have been carried out, HU exists as a heterodimer, composed of HU-α and HU-β, which are closely related subunits encoded by genes *hup*A and *hup*B, respectively. HU binds to nucleic acids without the aid of any cofactors or proteins. Early experiments using electron microscopy, nuclease protection, affinity chromatography and nitrocellulose filtration have shown that the protein interacts with RNA, and with single-stranded and double-stranded DNA without any sequence specificity. Electrophoretic mobility shift assays (EMSA) have shown that HU binds to linear double stranded DNA regardless of the sequence with low affinity (K_d ~2.5 μM) in 200mM salt with a modest enhancement in low salt conditions [81]. One dimer binds every 9 bp in a weak cooperative manner ($\omega = 30$), with a slight bending of the DNA upon several HU molecules binding the DNA [81-83]. Thus, when small plasmids are incubated with the protein and then relaxed with topoisomerase, there is modest level of supercoiling following deproteinization [84, 85]. This is also observed in HU-DNA complexes in the electron microscope [84]. HU can introduce circles readily of 60-100 bp duplex DNA, whereas duplex DNA less than ~150 bp in length otherwise resists circularization due to its inherent inflexibility [82, 86].

Agents that deform the helical axis should preferentially bind to DNA that is pre-bent [87]. This is observed in *E. coli* HU, where the affinity for the four-way junction is 1000-fold stronger than for linear DNA under stringent conditions. Two HU dimers bind to opposing sides

of the junction with no cooperativity (ω = 1) and high affinity (K_d= 4 nM). This binding is not inhibited by a 100-fold excess of linear DNA [70, 88]. HU also has high affinity for duplex DNA containing an interrupted motif such as a nick or a gap of one or two nucleotides [69]. Though these DNA structures are different, HU binds with high affinity (K_d ~8 nM, under stringent conditions) to both junction and discontinuous DNA compared to the non-specific and weak binding (K_d ~25,000 nM) under similar conditions to linear duplex DNA molecules [89].

Role of HU

In Mu Transposition

HU plays a pleiotrophic role in bacteria. It was first characterized as a histone-like protein in *E. coli* for its ability to introduce negative supercoils into relaxed DNA in the presence of topoisomerase I [90]. HU contributes to the maintenance of the intricate balance of DNA superhelical density by constraining DNA and modulating the topoisomerase I activity [91]. Efficient *in vitro* transposition of bacteriophage Mu requires HU [92], where it plays an essential role that leads to the formation of the stable synaptic complex (SSC, or type 0 transposome) in which multiple copies of the Mu transposome assemble to attach to target DNA during later stages of transposition. Fine mapping of co-localization of HU with Mu transposase at the donor ends was achieved by an elegant experiment in which HU was converted by chemical modification into a nuclease [93]. Later studies have shown one HU heterodimer binds around the center of two MuA binding sites by HU-induced DNA bending creating a footprint of ~30 bp [94]. HU can be displaced from the Mu transposome complex after assembly steps by high salt [93], but not so from the *gal* DNA by addition of heparin (25 μg/ml) or by chasing with excess HU [95], where HU functions as an accessory factor in transcriptional regulation.

In Transcription

In the Gal repressosome, a higher order nucleoprotein complex that represses transcription of *gal* operon in *E. coli*, two GalR dimer proteins bind to two operators, O_E and O_I, by forming a DNA loop of 113 bp around the promoter sequence [96-98]. The 113 bp DNA loop requires the architectural protein HU and supercoiled DNA for transcription initiation from *gal* promoters, *P*1 and *P*2 [99-101]. The repression is removed by binding of D-galactose to GalR [97]. HU specifically interacts with a segment of the DNA loop, centered at position +6.5, and to GalR [95]. Though the DNA loop closes by the tetramerization of the two DNA-bound GalR dimers without HU as an adaptor in between, the dimer-dimer interaction requires supercoiling of HU as an architectural protein during loop formation [102].

In DNA Inversion

HU also plays an accessory role in DNA inversion together with FIS and a recombinase, Hin, that promotes recombination between inversely repeated loci, the *hix* sites in *E. coli* and *Salmonella typhimurium* [103]. Fifteen to twenty HU dimers are required per molecule of DNA to form a loop between the enhancer and the nearby *hix* site, an amount sufficient to coat the 100 bp loop but not the entire plasmid. Cells depleted of HU are impaired of Hin-mediated recombination suggesting that no other bacterial protein can assist looping in this system [104].

In DNA Replication

HU is involved in initiation of DNA replication *in vitro* at the replication origin in *E. coli* [105]. HU has been located at or near this *in vitro* prepriming complex, formed by supercoiled plasmid DNA containing the chromosomal origin sequence *ori*C [106]. The dnaA, dnaB, dnaC and HU proteins assemble to form the large complex around *ori*C that seems to be wrapped around the dnaA protein as indicated by nuclease digestion studies [107]. The replication bubble

is formed upon addition of single-stranded DNA binding protein and DNA gyrase, which is recognized by *E. coli* primase as a template and thus initiates bidirectional replication by DNA polymerase III.

In DNA Repair and Recombination

HU, like high mobility group proteins, HMGB1, protects DNA against γ-ray and UV induced cleavage *in vitro* as seen in cells lacking HU having increased amounts of double-stranded breaks [108, 109]. It is associated with RecA and participates in homologous recombination repair [109-110].

Eukaryotic histones H1-H5 and proteins of the HMG class have low affinity for double-stranded DNA with no sequence-specificity, but high affinity for DNA junctions [111-112]. Another class of DNA junction-recognizing proteins, the resolvases and nucleases bind and cut DNA junctions without any sequence preference [113-114]. However, HU binds specifically to DNA with a nick or a gap and DNA junctions: one HU dimer binds to DNA containing a nick or a gap [69, 89], while two HU dimers bind specifically to DNA junctions [70, 88]. Phenanthroline protection analysis and Fe-EDTA footprinting shows HU binding to both the substrates and interacting with the minor groove. The protected region is large, ~20 bp, which can be explained by bending of the DNA upon HU binding [67]. Based on circular permutation assays, when one HU dimer binds to nicked DNA, a curvature of 65˚ is introduced at the break point between the two double helices of DNA [67]. Due to the presence of alternative pairing of junction branches, there is a symmetrical cleavage pattern with the junction DNA and not with nicked DNA. In DNA junctions HU reflects a slight preference for DNA sequences in strands C and D. The HU center of symmetry is juxtaposed on the 3′ side of strand C instead of the 3′ side of strand D (80% of the junctions), which is similar in nicked DNA (Figure 1.2). In both conformations, the

two dimers are on opposite sides of the junction and interact only via their β-arms separated by ~6Å, whereas interaction between the two bodies or the α-arms is not possible [67]. This is in agreement with the lack of cooperativity in the binding of first and second HU dimers to the DNA junction [89]. The position and orientation of HU heterodimer on the DNA show that the HU β-arm selectively binds the strand with the break and orientation of the arm is antiparallel to the broken strand (direction 3′ to 5′) and the center of symmetry of the dimer lies on the 3′ branch of the DNA (the branch containing the 3′ end of the broken strand) (Figure 1.2) [67]. The involvement of the HU body in the interaction with the nicked DNA explains the 100-fold stronger affinity of HU for nicked DNA compared to double-stranded DNA [69, 89].

As HU and IHF have similar three-dimensional structures, the position of HU and IHF is also very similar on the DNA that has a nick in the middle. However, the position of IHF on the nick is due to sequence specificity whereas for HU it is based on the structural features. Also, as IHF introduces two kinks of 80˚ into nicked DNA [52], HU introduces only one kink at the DNA break point, which allows contact with the 5′ arm of the DNA and the body of HU. Though HU can be substituted for IHF *in vitro* [115], *in vivo* production of IHF cannot compensate for HU in *hupAB* mutants [116].

Using magnetic tweezers and atomic force microscopy it has been observed that HU can have two opposing mechanical effects on DNA architecture depending on the protein concentration. At concentrations <100 nM, individual HU dimers induce flexible bends in DNA with compaction up to 50%, while at higher concentrations of HU, a rigid nucleoprotein filament forms with HU arranged helically around the DNA [117]. This stiffness may be due to binding of HU molecules out of phase with the DNA helical pitch, preventing the formation of the concerted structure or by destabilization of the protein scaffold by protein-protein interactions

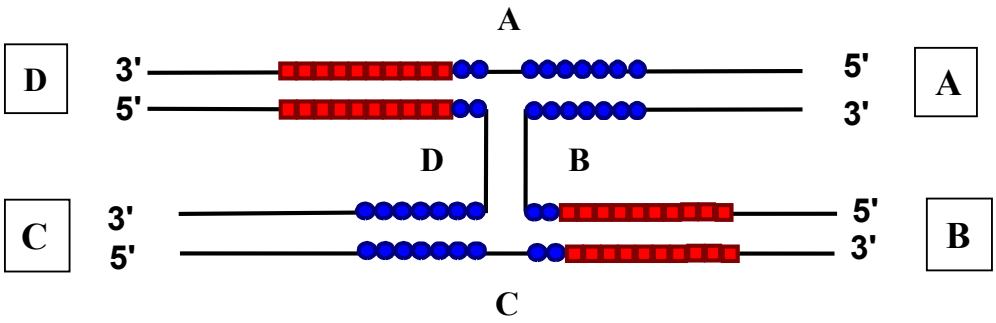

Figure **1.2**. Summary of binding pattern of *E. coli* HU to four way junction DNA with complex 1 binding to arms C and D and complex 2 binding to arms A and B. α-subunits in complex 1 and 2 are indicated in red squares and β-subunits in blue.

[118]. High affinity HU-DNA binding together with similar single molecule elasticity studies with IHF [119] indicates that histone-like proteins play an important role in shaping the bacterial nucleoid structure.

Background on *Deinococcus radiodurans*

Bacteria belonging to the family *Deinococcaceae* are some of the most radiation-resistant organisms discovered, and they are ubiquitous, vegetative, easily cultured and non-pathogenic [120-122]. Only eight members of this family have been studied and described [123]. *Deinococcus radiodurans* has been the most widely studied species for its accessibility for genetic manipulation and due to its natural transformability by both chromosomal DNA and plasmid DNA [124, 125]. *Deinococcus radiodurans* strain RI was the first deinobacterium to be discovered in Oregon in 1956 from canned meat that had been exposed to X-rays [126]. It is an aerobic, large (1-2 μm), red-pigmented (due to carotenoids present in the membrane), non-sporulating, mesophilic, Gram-positive coccus, capable of forming tetrads and best known for its extreme resistance to ionizing radiation, UV light, prolonged dessication, hydrogen peroxide, and numerous agents that can damage DNA [121, 127-128].

Phylogenetic analysis of Deinococcal 16S and 5S rRNA sequences indicates that *Deinococcus* is not related to *Micrococcus* as originally classified, but to Gram-negative *Thermus* (S_{AB} = 0.22 to 0.29) [129]. It is a widely distributed soil dwelling organism, found in organic rich environments like animal faeces, processed meats and sewage. Successful isolations of deinococci from dried foods, room dust, medical instruments, textiles and weathered granite of the Antarctic valley suggest its capacity to survive in dry, nutrient-poor surroundings [130].

The cell envelope is unusual in terms of chemical composition. Though it stains Gram-positive, its outer cellular layer is reminiscent of Gram-negative bacteria. The cell envelope is

comprised of plasma and outer membranes (formed of unconventional glyophospholipids), followed by a 14-16 nm peptidoglycan layer and an uncharacterized "compartmentalized" layer [131].

The genome has a base composition that is high in GC content ranging from 65-71 mol%. It is comprised of two chromosomes (DR_Main [2.65 Mbp] and DR412 [412 kbp]), one megaplasmid (DR177 [177 kbp]), and one plasmid (46 kbp), carrying 3,195 predicted genes [132]. The genome has 8-10 haploid copies during exponential growth and 4 copies during stationary phase. In comparison, *E. coli* has 4-5 haploid chromosomes during vigorous exponential growth [133]. In stationary phase, *D. radiodurans* does not die until exposed to 1.5 megarads of ionizing radiation, which represents more than 100-fold resistance compared to *E. coli*. In exponential phase it is 33-fold more resistant to UV than *E. coli* [121]. It can also survive in a desiccator (< 5% humidity) for up to 10 weeks with 85% viability, and anecdotal reports indicate it can survive in a dessicator for 6 years with 10% viability [134, 135]. It is suggested that *D. radiodurans* can reconstitute its genome from a high level of DNA damage of 1,000 to 2,000 double-strand breaks whereas the maximum capability of *E. coli* is 10 to 15 double-strand break fragments. Though the organism has an efficient DNA repair system, it does not provide an obvious answer to its extremely high capability for reconstitution of damaged DNA.

The ability to survive the potentially damaging effects of ionizing and ultraviolet radiation and desiccation has been suggested to be a combined effect of three mechanisms: prevention, tolerance and repair. The catalase and superoxide dismutase (SOD) mutants have increased sensitivity to ionizing radiation compared to wild type [136], indicating that prevention may be a contributing factor in the resistance of the organism. *D. radiodurans* also has a high number of predicted highly expressed (PHX) chaperone, detoxification, and protease genes

which can protect the cell from oxidative damage [137]. Scavenging of oxygen radicals in the presence of catalases, one of which is induced after exposure to ionizing radiation [138], multiple SOD and two homologs of DPS (Starvation inducible DNA-binding protein) protein in *E. coli* is suggestive of this role. However, the main component of resistance seems to be DNA repair. On exposure to 1.75 Mrads of ionizing radiation which leads to several hundred double strand breaks, the genome is restored in most cells in almost 24 hours without rearrangement or increased mutation frequency. It has a full potential of DNA repair enzymes: nucleotide excision repair (a UvrABCD system and a UVDE system), base excision repair (nine DNA glycosylases and an apurinic-apyrimidinic endonuclease), mismatch excision repair (MutL and MutS), and recombinational repair (RecA, RuvABC and SbcCD). However, photoreactivation and adaptive response to alkylation damage is not present [127]. All of the DNA repair genes identified in *D. radiodurans* have functional homologs in other prokaryotic species, suggesting that the complement of genes is not sufficient to explain the extreme resistance of this organism.

The limited information available suggests that survival of *D. radiodurans* is based on: (1) Homologous recombination which is initially a template-independent process that is independent of RecA protein and is facilitated by controlled diffusion of DNA fragments [139]. Template-dependent recombination involving the interchromosomal recombination in multiple chromosomes [140] is also expected to contribute to efficient double-strand break repair. Based on accurate mass tag measurements, RecA is detected in normal growth conditions, but elevated upon irradiation [141] and has been proposed to play a role in bringing together overlapping DNA fragments [142]. The recently solved crystal structure of RecR from *D. radiodurans* suggests its functional role in recombination, where the tetrameric ring in complex with RecF or

RecO may be functioning as a nonsliding DNA clamp capable of opening and closing the ends of the DNA [143].

(2) Post-irradiation DNA degradation to provide single stranded DNA for recombination. Initial studies indicate that the single strand DNA binding protein (SSB) from *D. radiodurans* promotes DNA strand exchange reactions by RecA from *D. radiodurans* and *E. coli* with the same efficiency as *E. coli* SSB homotetramer [144].

(3) Regulation of DNA replication and post-irradiation DNA degradation. DNA replication is held in check until repair is complete, suggesting these processes are coordinately regulated. A regulatory protein IrrI is activated shortly following DNA damage which limits the extent of DNA degradation following the action of endonuclease α [145]. The binding of this factor to DNA repeats is increased to a maximum after 3 hours of DNA damage and continued at the same level till 7 hours, and decreased gradually to uninduced levels after 24 hours.

(4) Export of damaged nucleotides from the cell to avoid mutation. UV and γ radiation induced DNA degradation forms oligonucleotides approximately 2,000 bp long and a mixture of damaged and undamaged nucleotides and nucleosides which are rapidly exported out of the cell into the cytoplasm and surrounding growth medium [146]. The extent of free-nucleotide removal is linearly related to the dose of radiation. The presence of two UvrA homologs (UvrA1 and UvrA2) involved in recognition of DNA damage for nucleotide excision repair [147] may be also closely related to ABC transporter proteins as other Uvr homologs. UvrA from *E. coli* also serves as a site for attachment of nucleotide excision repair proteins to the cell membrane [148]. UvrA2 may be a component of the nucleotide transporter complex, as it is very closely related to DrrC protein from *Streptomyces peucetius* which probably functions in transporting antibiotic

daunorubicin out of the cell [149]. The removal of the nucleotides from the cell is possibly a part of the signal which coordinates the DNA repair cascade.

Deinococcus encodes an ortholog of the chromosomal DNA-binding protein HU believed to play a role in DNA packaging and as a cofactor in recombination [150]. As a complete understanding of the DNA repair mechanism is hard to configure in this repair-proficient organism, we initiated studies related to the role of this protein. The gene was cloned from the genomic DNA and the protein was purified and characterized in terms of its *in vitro* DNA-binding capabilities. The protein has distinct properties in comparison to other HU homologs, properties that are in part conferred by its unique N-terminal extension. For comparison, studies have also been conducted with a HU homolog from another mesophilic organism, *Helicobacter pylori*, to analyze its stability and role in recombination. These studies indicate that the HU homolog from *D. radiodurans* is unique amongst members of the HU family, and its DNA binding properties suggest its role in DNA recombination.

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CHAPTER 2

HISTONE-LIKE PROTEIN HU FROM *DEINOCOCCUS RADIODURANS* BINDS PREFERENTIALLY TO FOUR-WAY DNA JUNCTIONS

Introduction

Prokaryotes synthesize several proteins whose primary function is to compact and organize the genomic DNA [1-3]. Of these so-called histone-like proteins, HU is the most abundant. Ubiquitous dimeric proteins, usually composed of 90-99 amino acid residues, HU proteins exhibit significant sequence and structural homology. The structure of the homodimeric *Bacillus stearothermophilus* HU reveals that the two monomers form a relatively compact body from which two β-sheet 'arms' extend [4-6]. The structure of the homologous Integration Host Factor (IHF) from *Escherichia coli* in complex with DNA shows that highly conserved prolines at position 63 are positioned at the tips of the DNA-embracing β-sheets to intercalate into the base pair stack to form two sharp kinks totaling ~160°. Notably, *Anabaena* HU introduces a variable bend angle of ~105-140° suggesting that the site of DNA distortion is best modeled as a hinge and not a rigid bend [7, 8]. As demonstrated by DNA-binding experiments and as seen in the two co-crystal structures, the two DNA kinks are introduced at a separation of 9 bp [7-10].

In *E. coli*, where it has been most widely studied, HU coils DNA into nucleosome-like structures [11]. *E. coli* HU is an architectural protein that binds non-specifically and with low affinity to double-stranded DNA and with nM affinity to distorted DNA to cause significant DNA bending, negative supercoiling and DNA compaction [12-17]. Cells lacking HU are highly sensitive to γ and UV irradiation, suggesting its role in DNA repair and recombination [18-21].

The DNA-binding sites for sequence-specific HU homologs, *E. coli* IHF and the *B. subtilis* bacteriophage SPO1-encoded TF1, are ~35 bp, with electrostatic interactions between DNA phosphates and the DNA-binding surface serving to wrap the DNA around the body of the protein [7, 10]. The *Thermotoga maritima*-encoded HU homolog also has an optimum binding site of ~37 bp, and it binds duplex DNA with nM affinity [22]. In contrast, *E. coli* HU has been reported to bind a much shorter ~9 bp DNA target with a relatively low (~300 nM) affinity [16, 23]. Notably, HU homologs encoded by *Rhizobium leguminosarum* and *Borrelia burgdorferi* exhibit sequence-specific DNA binding [24, 25]. Taken together, the wide range of substrate selectivity reported for HU homologs suggest that their binding properties are optimized for specialized cellular functions in individual organisms.

Deinococcus radiodurans has extremely high tolerance for the DNA double strand breaks that result from ionizing radiation or prolonged desiccation [26, 27]. After exposure to irradiation, *D. radiodurans* can reconstitute its genome from 1,000 to 2,000 double strand break fragments, whereas *E. coli* can restore its genome from no more than 10-15 fragments (for review, see 28). The annotated *D. radiodurans* genome has left few clues to this extraordinary capacity for reconstituting a fragmented genome [29].

We show here that *D. radiodurans* HU (DrHU) binds double-stranded DNA with moderate affinity, but with significant preference only for pre-bent DNA, such as four-way junction structures. Notably, DrHU does not exhibit marked preference for DNA with flexure points such as loops or gaps. We propose that binding properties of DrHU are optimized for participation in recombination events. Our data also suggest that DNA–binding properties of HU homologs are defined by their ability to stabilize DNA bends.

Materials and Methods

Cloning, Overexpression and Purification of DrHU

The gene encoding DrHU was amplified from *D. radiodurans* RI genomic DNA generously provided by J. Battista. Primers DrHU–up (5'-GCTCTATCCCCATATGCCCCCCTTCACAGC-3') and DrHU-down (5'-CGGAGGGAGCGGTCACATATGAACCCGCTTACAGG-3') were designed according to the genomic DNA sequence (gi: 15807733 [29]) and modified to introduce *NdeI* sites at both ends of the PCR product (underlined in the primer sequences). The PCR product was cloned into pET5a, generating plasmid pET-DrHU. Integrity of the construct was confirmed by sequencing. Plasmid pET-DrHU was transformed into *E. coli* BL21(DE3)pLysS and overexpression initiated by addition of IPTG to a final concentration of 1 mM. Cells were harvested two hours after induction and stored at –80°C.

All steps of DrHU purification were carried out at 0°C to 4°C. Cells were resuspended in lysis buffer (50 mM Tris-HCl (pH 8.0), 0.25 M NaCl, 5 mM Na_2EDTA, 5% (v/v) glycerol, 5mM 2-mercaptoethanol, 0.1mM phenyl methyl sulfonyl fluoride (PMSF)) and lysed by sonication. Polymin P (BASF) was added dropwise from a 13% (v/v) solution to a final concentration of 0.5%. The cell lysate was centrifuged at 12,000 g for 20 min and the supernatant was slowly adjusted to 40% saturation with $(NH_4)_2SO_4$, stirred for 30 minutes, centrifuged at 15,000 *g* for 10 minutes, and the sediment discarded. Solid $(NH_4)_2SO_4$ was added to 75% saturation and the precipitate forming during 30 minutes of stirring was collected, dissolved in buffer A (20 mM Tris-HCl pH 8.8, 50 mM KCl, 5% glycerol, 1 mM Na_2EDTA, 3.5 mM 2-mercaptoethanol, and 0.2 mM PMSF), dialyzed against buffer A, and applied to a CM-Sepharose column equilibrated in buffer A. The protein was eluted with a linear gradient of 50 mM to 1M KCl in Buffer A. Peak

fractions were adjusted to 40% saturation with $(NH_4)_2SO_4$ while stirring and applied to a phenyl-Sepharose column equilibrated in Buffer A containing 40% $(NH_4)_2SO_4$. DrHU was eluted with a linear gradient of 40% to 0% $(NH_4)_2SO_4$. Peak fractions were pooled and dialyzed against Buffer A at pH 8.0 and loaded on a hydroxyapatite column equilibrated in Buffer A at pH 8.0, and eluted as described for the CM-Sepharose column. Peak fractions were pooled and dialyzed in Buffer A pH 9.6, and applied to an equilibrated Heparin-Sepharose column in buffer A at pH 9.6, and eluted as described for the CM-Sepharose column. Purity was ascertained by both Coomassie Blue and silver staining of SDS/polyacrylamide gels. Protein concentrations were determined by quantitation of Coomassie blue stained SDS-polyacrylamide gels, using BSA as a standard.

Cross-linking of DrHU

DrHU was incubated with 0.1% glutaraldehyde in 10 mM sodium phosphate, pH 7.0, at room temperature for 30 min. Samples were diluted 1:2 with Laemmli sample buffer, and analyzed by electrophoresis on 17% SDS/polyacrylamide gels followed by Coomassie Blue staining.

Preparation and Labeling of DNA Probes

Oligonucleotides were purchased and purified by denaturing polyacrylamide gel electrophoresis. The top strand, shared among loop-, nick-, gap- or bulge-containing duplexes, was ^{32}P-labeled at the 5'-end with phage T4 polynucleotide kinase. Equimolar amounts of complementary oligonucleotides were mixed, heated to 90ºC and cooled slowly to 4°C to form duplex DNA. Nicked and gapped constructs were generated by annealing two oligonucleotides (terminating with free hydroxyl group) to the top strand. Annealing a 33 nt oligonucleotide to the top strand generated a 3'-overhang of 4 nt. The construct with bulges was made by annealing an

oligonucleotide with a set of three Cs inserted at a spacing of 9 bp to the top strand. The sequence of the G+C-rich 37-mer, selected randomly from the Megaplasmid of *D. radiodurans* was 5'-CCCCGTCTGTCCCCCGATCCCCTGCTCGTAGGCGTG-3'. Each strand of the 89-mer DNA constructs was generated by ligation of two shorter oligonucleotides, using a complimentary oligonucleotide to direct apposition of the two oligonucleotides forming the full-length strand. Top and bottom strands were purified by denaturing polyacrylamide gel electrophoresis, and complementary strands were annealed as described above to form the 89-mer duplex DNA or duplex with loops, nicks or gaps. The sequence of the 37 bp duplex (Figure 2.3) corresponds to the T-containing version of the preferred binding site for the HU homolog TF1 that overlaps the bacteriophage SPO1 early promoter P_E6 [10]. The 50, 60 and 89 bp duplexes were generated by extending the 37 bp DNA at either end with surrounding SPO1 sequence, except for GC base pairs at either end that were introduced to reduce fraying (sequences available on request).

Four-way junctions (4WJ) were constructed as described [13] using the following oligonucleotides:

strand 1, 5'-CCCTATAACCCCTGCATTGAATTCCAGTCTGATAA-3'

strand 2, 5'-GTAGTCGTGATAGGTGCAGGGGTTATAGGG-3'

strand 3, 5'-AACAGTAGCTCTTATTCGAGCTCGCGCCCTATCACGACTA-3'

strand 4, 5'-TTTATCAGACTGGAATTCAAGCGCGAGCTCGAATAAGAGCTACTGT-3'.

The 4WJs were prepared by annealing of strands 1-4, followed by purification of the junctions on nondenaturing polyacrylamide gels. The identity of the 4WJs was verified by separation of aliquots of gel purified junctions (subsequently ^{32}P-labeled at the 5' termini) on a denaturing gel.

Linear plasmid DNA was prepared by linearizing pET5a (Novagen) with *Eco*RI. Nicked plasmid was prepared by incubation of pET5a with N*Bst*N1 (New England Biolabs).

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays were performed using 8% (w/v) polyacrylamide gels (39:1(w/w) acrylamide:bisacrylamide) in TBE (45 mM Tris-borate pH 8.3, 1 mM EDTA). Gels were prerun for 30 minutes at 20 mA at room temperature before loading the samples with the power on, except for experiments with bulged DNA which were run at 4°C. Reaction conditions were as described [30], and each sample contained 100 fmol DNA in a total volume of 10 μl unless indicated otherwise. After electrophoresis, gels were dried and protein-DNA complexes were visualized and quantified by phosphorimaging. The region on the gels between the complex and free DNA was considered as complex.

Binding isotherms were evaluated by non-linear fits of DNA titrated with protein, using the equation $Y=(Y_{max}*X)/(K_d+X)$ where K_d is the observed equilibrium dissociation constant, assuming protein binding to a single site, Y is fractional complex formation, and X is protein concentration. A modified version of the McGhee-von Hippel binding isotherm for nonspecific binding to a finite DNA lattice was also used [36, 37]. Complex dissociation during electrophoresis was measured as described [10] and the observed fraction of complex Y(t) was corrected according to the equation $Y_{corr} = Y(t)/\exp(-k_{diss}\cdot t)$ where t is time of electrophoresis and k_{diss} is the exponential decay constant for complex dissociation during electrophoresis. All experiments were carried out at least in triplicate. K_d values are reported as the average ± the standard error of the mean.

Analysis of binding to plasmid DNA was performed using 0.5% agarose gels in TBE or TAE (40 mM Tris acetate, 2 mM EDTA). Reaction conditions were as described above, and each

sample contained 25 ng DNA (linear, nicked or supercoiled template) in a total reaction volume of 15 μl. Gels were stained using ethidium bromide.

Two-dimensional Methidiumpropyl-EDTA (MPE)-Fe(II) Footprinting

DNA probes were prepared by 5'-end-labeling one strand prior to annealing the complementary strands of the 4WJ or the 37 bp bulged DNA. The 4WJ was purified on a 10% polyacrylamide gel. Protein-DNA complexes were formed in a 10 μl volume for 5 min at room temperature under reaction conditions as described above. Each reaction contained 0.5 pmoles of DNA. 1 μl of 10 mM sodium ascorbate and 2 μl of freshly prepared 25 μM MPE-Fe(II) was added to each reaction which was terminated after two minutes at 4ºC by loading samples onto running non-denaturing 8% polyacrylamide gel in TBE. Protein-DNA complexes were isolated, and the DNA was passively eluted from gel slices, extracted with phenol-chloroform, precipitated with ethanol, and resolved on 15% polyacrylamide sequencing gels. Density profiles were obtained by phosphorimaging.

Supercoiling Assays

Negatively supercoiled pUC18 or pGEM5 (100 ng/reaction) was relaxed in 10 mM Tris-HCl, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA with *Vaccinia* DNA topoisomerase I (50 U/μg DNA; Epicentre) at 37°C for 120 min. The relaxed DNA was incubated with increasing amounts of protein and the volume adjusted with 1X dilution buffer (20 mM Tris-HCl pH 7.5, 0.1 mM Na_2EDTA, 0.05% (w/v) Brij58, 100 μg/ml BSA). Reactions were allowed to proceed at 37°C for 1h, after which 5 μl of the termination mix (5XTBE, 5% SDS, 15% sucrose, 0.1% bromophenol blue, 0.1% xylene cyanol, 2 μg/μl proteinase K) was added, and the samples were subjected to further incubation at 37°C for 1h.

DNA Circularization

Plasmid pET5a was digested with *Bsp*HI to yield a 105 bp fragment, which was purified on a 2% agarose gel. The 136 bp DNA was made by digestion of pET5a with *Bam*HI and *Bgl*II. To prepare a nicked 105 bp fragment, a N*Bst*N1 site was introduced in the middle of the 105 bp fragment by PCR amplification of pET5a using a forward primer designed to introduce the N*Bst*N1 site. The reaction generated full-length plasmid harboring the N*Bst*N1 site. The original pET5a template was removed by *Dpn*I digestion, and the plasmid with the mutated sequence was used to transform *E. coli* TOP10 (Invitrogen). The sequence of the mutated plasmid was confirmed by sequencing. The plasmid DNA was digested with N*Bst*N1 and *Bsp*HI. The nicked 105 bp fragment was gel purified and ^{32}P-labeled at the 5'-end with phage T4 polynucleotide kinase.

Time-course ligation experiments and experiments with increasing protein concentrations were carried out for the cyclization kinetics studies [41]. Reactions were initiated by addition of 80 U of T4 DNA ligase to a final volume of 10 μl. Reactions containing 100 fmol DNA and the desired concentration of DrHU were incubated in 1X binding buffer (20 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, 0.1mM Na_2EDTA, 0.1 mM DTT, 0.05% (w/v) Brij58, 100 μg/ml BSA) with 1X ligase buffer (New England Biolabs) and 0.5 mM ATP at room temperature for the desired time and terminated with 5 μl of 75 mM EDTA, 3 mg/ml proteinase K, 15% glycerol, and bromophenol blue and xylene cyanol, followed by a 15 min incubation at 55°C. Reactions were analyzed on 8% polyacrylamide gels (39:1(w/w) acrylamide: bisacrylamide) in TBE. After electrophoresis, gels were dried and ligation products visualized and quantified by phosphorimaging.

Results

DrHU has a unique 47 amino acid extension at the N-terminus, followed by the DNA-binding fold characteristic of type II DNA-binding proteins (Figure 2.1). The DNA-intercalating Pro at position 63 of *B. subtilis* HU is conserved, but the surrounding residues are divergent in the *Deinococcus* and *Thermus* groups where Arg at position 61 is replaced by Val, and Asn at position 62 is replaced by Arg or Lys [22]. The presence of Gly at position 15, in the loop connecting helices 1 and 2, is noteworthy as Gly15 is otherwise primarily found in HU from thermophiles (e.g. *B. stearothermophilus* and *Thermotoga*) where it has been shown to contribute to loop flexibility and thermostability [30-33]. The presence of Gly15 in the loop connecting helices 1 and 2 in DrHU may likewise confer enhanced thermal stability.

DrHU was cloned from the genomic DNA of *D. radiodurans* RI and purified to ~95% homogeneity (Figure 2.2). DrHU exists predominantly as a dimer in solution, as shown by glutaraldehyde-mediated crosslinking. At protein concentrations >20 μM, primarily higher order aggregates exist which fail to enter the gel; this aggregation is reversible, as dilution from concentrated stock solutions results in mainly dimeric DrHU.

DrHU Binds Preferentially to Pre-bent DNA

To analyze DNA binding by DrHU, we used a 37-bp duplex DNA, which represents the longest reported binding site for an HU homolog (Figure 2.3). To determine whether DrHU, like other HU proteins, produces two DNA kinks with a 9 bp separation, we also prepared DNA constructs containing a set of 4-nt loops with a 9 bp separation [10].

Electrophoretic mobility shift assays (EMSA) were performed using concentrations of DrHU <1 μM, more than 10-fold lower than the concentration at which higher-order aggregates predominate. No stable complex formed on the 37 bp duplex (Figure 2.4A) whereas one faint,

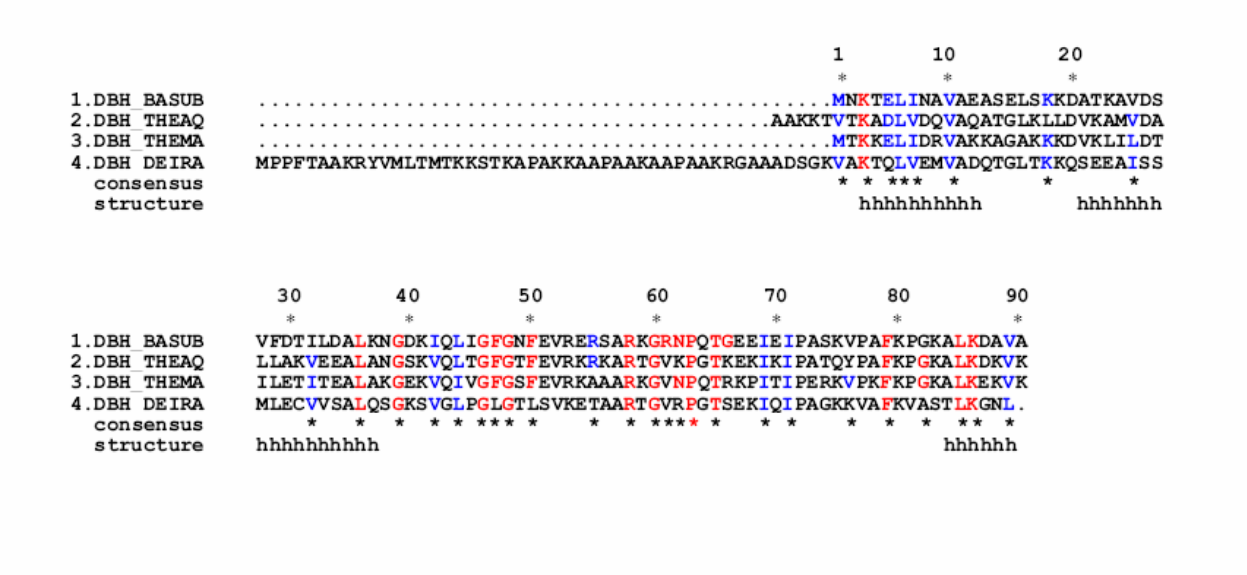


Figure **2.1**. Sequence conservation of the DNA-binding fold of DrHU. Residues are numbered based on the sequence of *B. subtilis* HU. Residues that are more than 80% identical among 60 originally aligned sequences are indicated in red, and residues that are more than 80% conserved are colored blue.[22] Asterisks below the alignment indicate conserved residues. The leftmost column identifies each protein (accession number in brackets): 1, HU, *Bacillus subtilis* (sp|**P08821**); 2, HU, *Thermus aquaticus* (PIR|**S12888**); 3, HU, *T. maritima* (sp|**P36206**); 4, HU, *D. radiodurans* (gb|**AAF12250.1**).

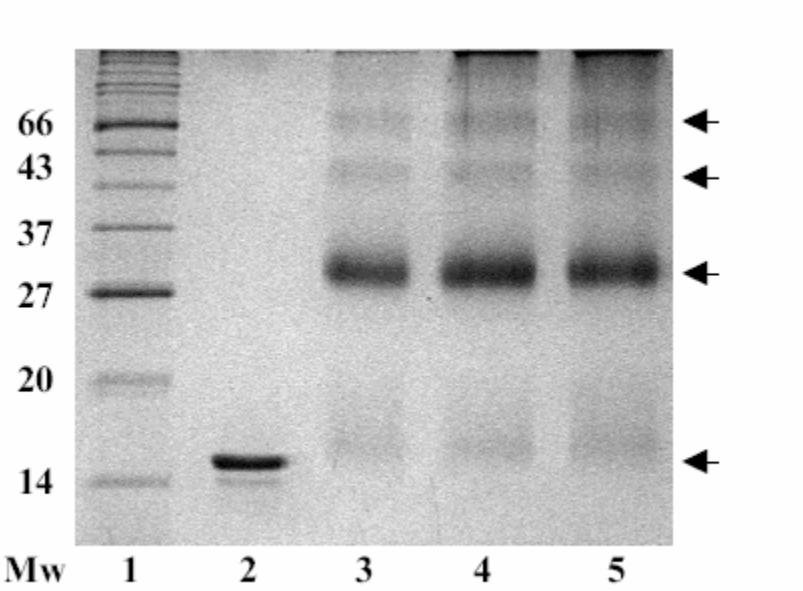


Figure **2.2**. DrHU exists as a dimer in solution. Lane 1, molecular weight marker; lane 2, 1 μg unmodified DrHU; lanes 3-5, 3 μg, 5 μg and 7 μg DrHU crosslinked with glutaraldehyde. DrHU has a molecular weight of 14,010 Da but migrates on the SDS-polyacrylamide gels at ~17 kDa due to its pI of 11. Molecular markers are in kDa. Arrows identify DrHU in different multimeric forms.

yet distinct DrHU-DNA complex was detected on DNA with loops (data not shown), indicating that DrHU does adopt the binding mode characteristic of other HU proteins, involving intercalation at two sites separated by 9 bp of duplex. As the G+C-content of *D. radiodurans* genomic DNA is very high, ~67%, binding of DrHU to a G+C-rich 37 bp duplex was also assessed. DrHU binds only slightly better to G+C-rich DNA compared to DNA of average G+C-content, forming only faint, unstable complex with the G+C-rich DNA (data not shown).

Incubation of DrHU with nicked 37 bp DNA of average G+C-content also results in the formation of only a faint complex (Figure 2.4B), and DrHU fails to form any detectable complex with DNA containing a 1-nt gap (Figure 2.4C) or a 2-nt gap (data not shown). Increasing the flexibility of DNA by introducing exocyclic substituents, for instance by replacement of thymine with 5-hydroxymethyl uracil (hmU), has also been shown to promote binding of DNA-bending proteins [9, 10]. An hmU-containing 37 bp duplex DNA in which all thymines are replaced with hmU show only a slight increase in affinity compared to T-containing DNA, comparable to that seen with nick- or loop- containing DNA (Figure 2.4B and data not shown). The affinity of DrHU for DNA with a 4-nt 3'-overhang is also low, and no stable complex is seen (data not shown). This is in distinct contrast to *E. coli* HU which was reported to bind DNA with nicks or gaps with at least 100-fold greater affinity compared to linear duplex DNA [16]. DrHU has significantly higher affinity for DNA with a set of bulge-loops separated by 9 bp, with an observed dissociation constant $K_d = 128 \pm 17$ nM (Figure 2.4D). This DNA construct may adopt a nearly planar U-shaped conformation, inferred by comparison to previously analyzed DNA molecules containing two dA_5-bulges separated by 9 bp of duplex [34]. Evidently, DrHU binds preferentially only to DNA with predisposed bends suggesting an ability to engage DNA stably only when the energetic cost of bending has been significantly lessened. Although this probe is

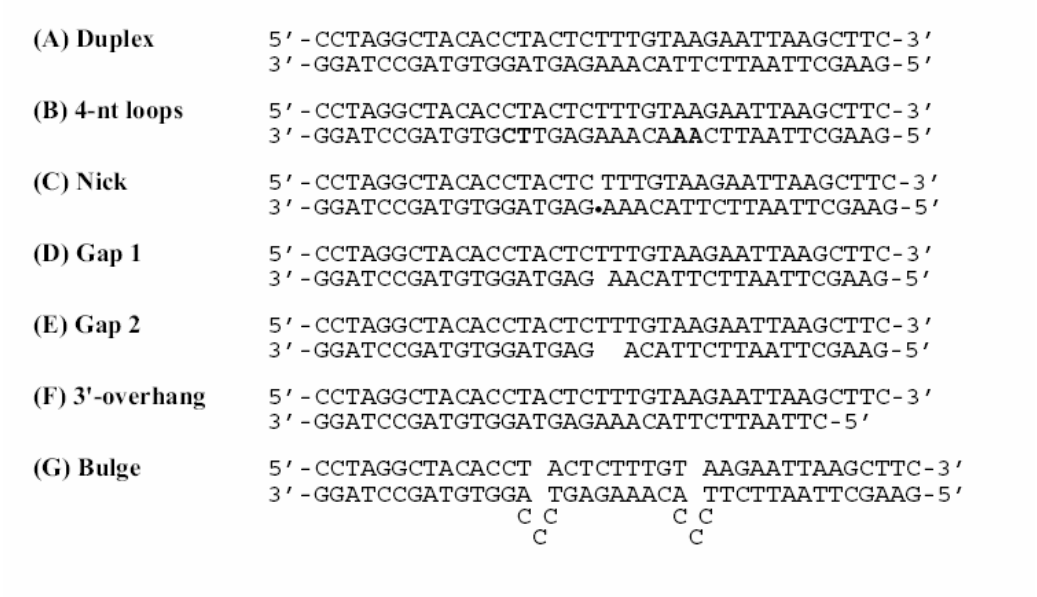


Figure **2.3**. 37 bp DNA constructs. (A) The sequence of the perfect duplex corresponds to the T-containing version of a preferred binding site for HU-homolog TF1. (B) In loop containing duplex, the sequence of the bottom strand is modified to generate tandem mismatches of identical opposing nucleotides with a spacing of 9 bp. Sequences generating loops are in boldface. (C) The nicked template is formed by annealing two oligonucleotides to the top strand. The position of the nick is indicated by a dot. Sequences that form the 1-nt gap, 2-nt gap and 3'-overhang are shown in (D), (E), and (F). (G) The bulged construct is made using a bottom strand containing a set of three C's at a spacing of 9 bp.

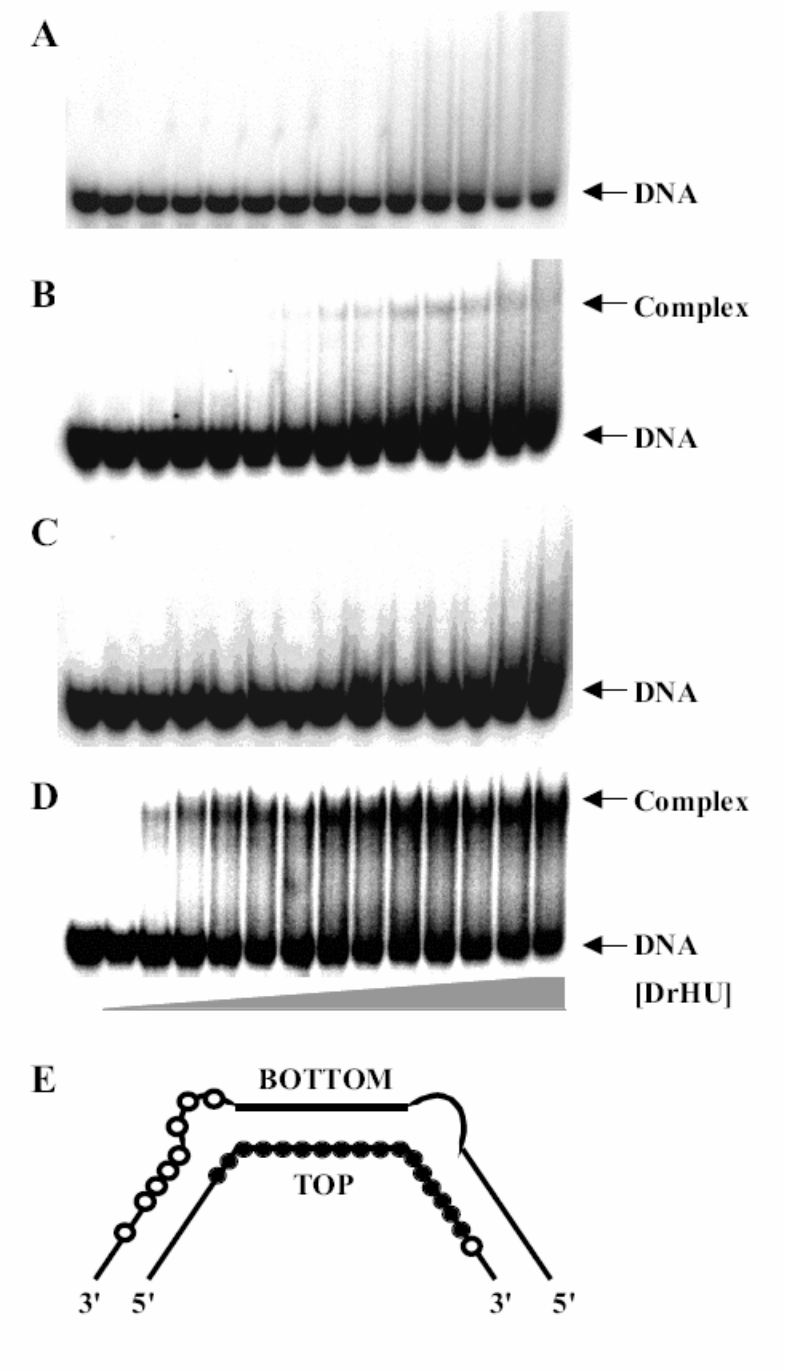


Figure **2.4**. DrHU binds preferentially to pre-bent DNA. Electrophoretic analysis of DrHU with (A) 37 bp duplex DNA (B) nicked DNA (C) 1-nt gap DNA (D) DNA with bulges. Complexes are indicated at the right; DrHU concentrations, identical for all panels, are 25-600 nM. (E). Summary of MPE-Fe(II) cleavage pattern for bulged DNA with (●) representing protection and (❍) indicating enhanced cleavage. Cleavage within ~12 nt of each 5'-end is not detectable as these short fragments are lost during isolation of the protein-DNA complex.

too short to permit determination of the complete protein footprint, it allowed confirmation of specific binding of DrHU by two-dimensional methidiumpropyl (MPE) Fe(II) footprinting (the lack of preference of DrHU for nicked DNA prevents redistribution of protein following DNA cleavage) [35]. DrHU protects the shorter top strand almost completely in the 9 bp region between the bulges and ~6 bp (about half a helical turn) downstream of the second bulge (Figure 2.4E). Even in the unbound DNA, the region between the bulges appears to be distorted as evidenced by reduced cleavage of the bulged-out bottom strand; in complex with DrHU, enhanced cleavage is seen at the second bulge and ~6 bp downstream. The pattern of protection and enhancement confirms specific binding of DrHU to this DNA construct, and it is consistent with placement of DrHU inside a U-shaped DNA construct that leaves the bulged-out bottom strand exposed to enhanced cleavage while the top strand is facing the protein.

As DrHU did not form stable complex with 37 duplex DNA, its binding to longer DNA templates was explored. The 50-mer, 60-mer and 89-mer were generated by nearly equal extension of both ends of the 37-mer duplex. With a 50 bp template, two DrHU-DNA complexes are seen (Figure 2.5A). The binding isotherm is hyperbolic, indicating that DrHU binding exhibits little or no cooperativity. The affinity is moderate, with a midpoint of the binding isotherm of 158 ± 26 nM (Figure 2.5B). No significant increase in affinity was seen with 60 bp DNA; likewise, DrHU does not have significantly higher affinity for a 50 bp G+C-rich template in comparison to the 50-mer DNA of average G+C content (data not shown). For analysis of DrHU binding, the modified McGhee-von Hippel equation that considers non-specific binding to a DNA duplex of finite length was applied [36, 37]. However, fits to the non-cooperative McGhee-von Hippel equation failed to converge. We surmise that linked equilibria involving dissociation of higher-order protein assemblies invalidate this binding model.

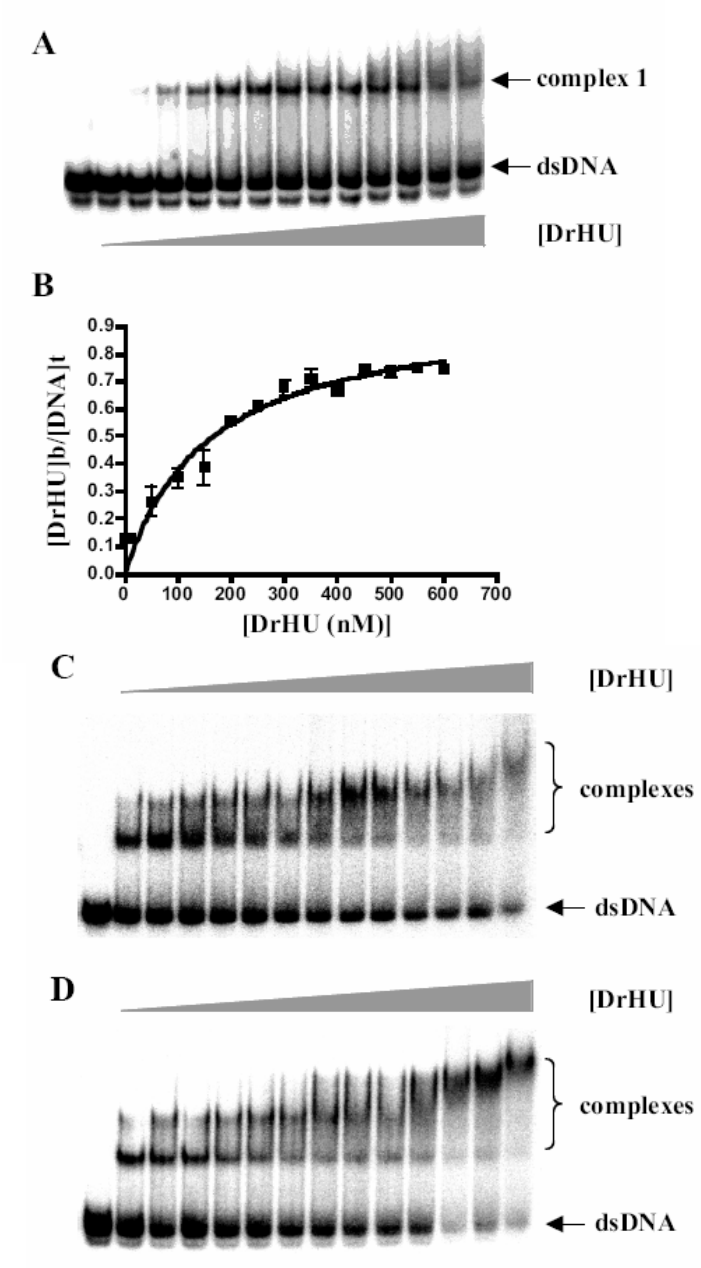


Figure **2.5**. Binding of DrHU to 50 bp and 89 bp DNA. (A) Electrophoretic analysis of duplex DNA titrated with DrHU. Complex one is indicated at the right, with a second unstable complex suggested by the smearing towards the top of the gel. Protein concentrations are 10-600 nM. (B) Ratio of bound DrHU to total [DNA] as a function of [DrHU]. C-D, Electrophoretic analysis of (C), 89 bp perfect duplex DNA and (D) 89 bp duplex with a central 1nt gap, both titrated with DrHU. Complexes are indicated at the right. Protein concentrations are 20-300 nM in C, and 10-300 nM in D.

DrHU binding to 89 bp duplexes was analyzed. The 89-mer was prepared as perfect duplex and as duplex with a set of 4 loops separated by 9 bp; the loop position is the same as for the 37-mer (Figure 2.3), placing the loops symmetrically about the center of the DNA. Three complexes of distinct mobility are observed (Figure 2.5C). Likewise, three complexes form on the 89-mer with loops, the set of 4-nt loops conferring only a very modest increase in complex formation (data not shown). The 89-mer DNA constructs with nick, 1-nt gap, 2-nt gap, and 5'- or 3'- overhangs also yield no significant increase in the affinity of DrHU compared to perfect duplex DNA (Figure 2.5 D and data not shown). In distinct contrast to *E. coli* HU, DrHU evidently does not bind preferentially to DNA with increased flexure, e.g. as conferred by nicks or gaps.

DrHU Binds Preferentially to Four-way DNA Junction

E. coli HU binds preferentially to 4-way junctions, consistent with its role in DNA inversion, recombination and repair [13, 16, 19-21]. We constructed a 4-way junction DNA (4WJ) based on the sequence used for analysis of *E. coli* HU [13]. A stable DrHU complex is formed at low protein concentrations, and a second complex is seen at higher concentrations (Figure 2.6 A). No evidence of cooperativity of binding is observed for DrHU, which binds the 4WJ with half-maximal saturation at 18.4 ± 2 nM (Figure 2.6B). Omission of $MgCl_2$ from the binding reaction resulted in a modestly higher affinity (12.5 ± 2 nM). Linear duplex DNA corresponding to two arms of the four-way junction, a 35-mer and a 40-mer respectively, were unable to form stable complex with DrHU, indicating a high specificity ratio (data not shown).

The position of DrHU on the 4-way junction was assessed by two-dimensional MPE-Fe(II) footprinting [35]. The 4WJs can exhibit either a stacked X conformation or an open,

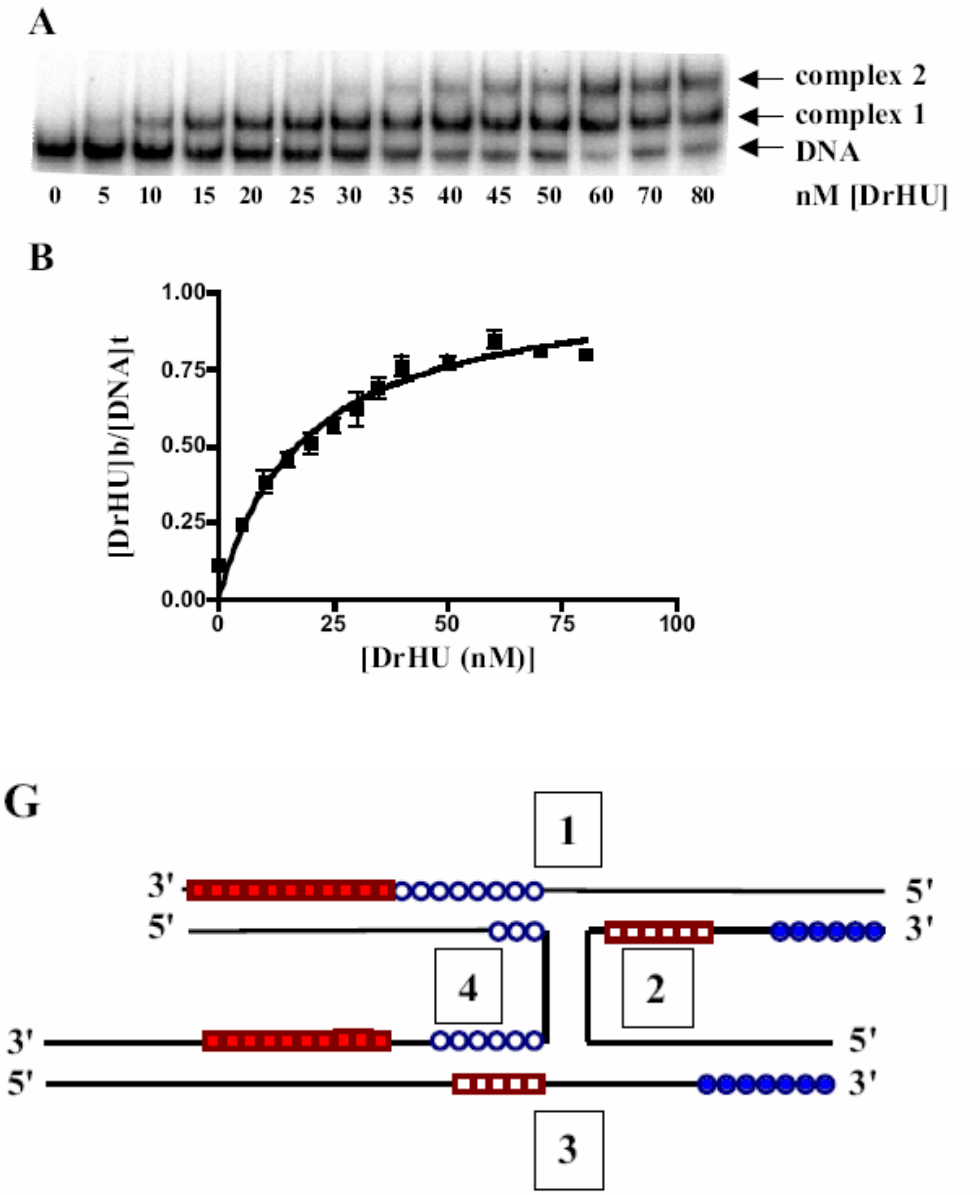
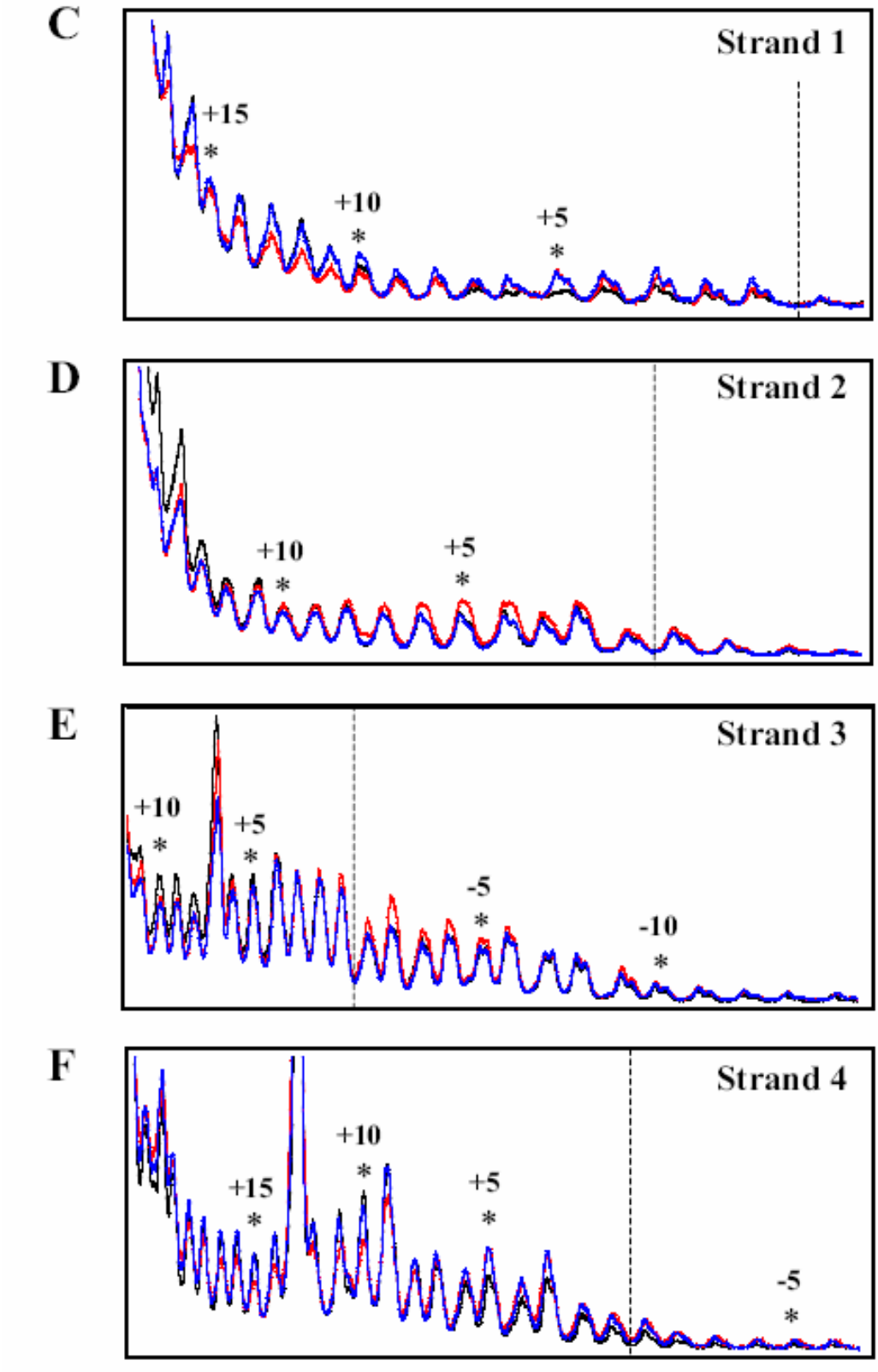


Figure **2.6**. Binding of DrHU to 4-way junction. (A) Electrophoretic analysis of DrHU with increasing concentrations of DrHU indicated below each lane. Each reaction contained 1.0 nM DNA. (B) Binding isotherm for DrHU binding to 4WJ DNA. (C)-(F) MPE-Fe(II) footprinting. Densitometric profiles corresponding to complex 1 (blue) and complex 2 (red) on strands 1-4, respectively. Free DNA is in black. (G) Summary of cleavage pattern with (●) representing protection and (❍) indicating enhanced cleavage in complex 1, and (■) and (□) indicating protection and enhancement, respectively, in complex 2. The vertical dotted line identifies the crossover, with positive and negative numbers identifying positions downstream and upstream of the crossover, respectively. (figure continued)


C
Strand 1
+15
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square conformation in which the junction arms are splayed out towards four corners, depending on the presence of metal ions [38]. We assayed binding to the stacked X conformation, favored in the presence of Mg^{++}; this conformation is characterized by antiparallel alignment of DNA strands, generating an interduplex angle of 40-60° [39-40]. The footprint corresponding to complex 1 revealed regions of incomplete protection and enhancement, as demonstrated by comparison of densitometric profiles (Figure 2.6 C-F). Protection of the four strands is asymmetrical, with primary regions of protection on strands 2 and 3 downstream of the crossover and concomitant enhancements at the crossover on strands 1 and 4 (Figure 2.6 G). This is indicative not only of specific binding of DrHU to the junction structure, but also of preferential binding to one pair of junction arms. While the predominant junction conformation in the absence of DrHU is predicted to be a stacked X, our data do not rule out that this conformation is altered upon protein binding. For complex 2, binding to the other pair of junction arms is indicated by protections on strands 1 and 4 downstream of the crossover, combined with enhancements on strands 2 and 3 close to the crossover. The pattern of protection and enhancement indicate specific binding of DrHU to a pair of junction arms while leaving the crossover exposed.

Supercoiling of Plasmid DNA

Analysis of DrHU binding to plasmid DNA (pET5a) showed a modest preference for supercoiled DNA, with no significant difference in protein binding to nicked and linear DNA (data not shown). We also note that these assays show no evidence of DNA compaction by DrHU, which would be manifest as an increased mobility of DNA in presence of DrHU, particularly evident with nicked DNA.

To assess the ability of DrHU to supercoil DNA, DrHU was incubated with covalently closed relaxed DNA and topoisomerase I. DrHU was modestly active in this assay, yielding a lower superhelical density compared to equivalent concentrations of B. subtilis HU (HBsu; Figure 2.7).

DrHU Does Not Bend Duplex DNA

We assessed the ability of DrHU to bend DNA using a circularization assay in which DNA shorter than the persistence length is circularized with T4 DNA ligase; the presence of a DNA-bending protein is evidence by an increased rate of ligase-mediated DNA circularization [41]. Surprisingly, DrHU does not circularize 105 bp DNA, nor does it circularize the linear multimers, indicating that it does not introduce a DNA bend (Figure 2.8). To assess if DrHU can enhance circularization with a more flexible DNA, a nicked 105 bp DNA was used. On incubation of nicked 105 bp template with increasing concentrations of DrHU and T4 DNA ligase, DNA circularization was still not observed (not shown). A longer 136 bp DNA circularizes in the presence of T4 DNA ligase, but the rate of circularization remains unchanged in the presence of DrHU (data not shown). Evidently, DrHU does not introduce a significant DNA bend when binding to either nicked or perfect duplex DNA, in notable contrast to other HU homologs.

Discussion

HU proteins are known primarily from analysis of the *E. coli*- and *B. subtilis*-encoded homologs, with *in vitro* properties of *E. coli* HU by far the most thoroughly studied [13, 14, 16, 23, 42]. The sequence conservation and structural homology between HU homologs led to the anticipation that DNA binding properties, and associated *in vivo* functions, would be similar for other members of this protein family. Accordingly, architectural roles involving DNA bending

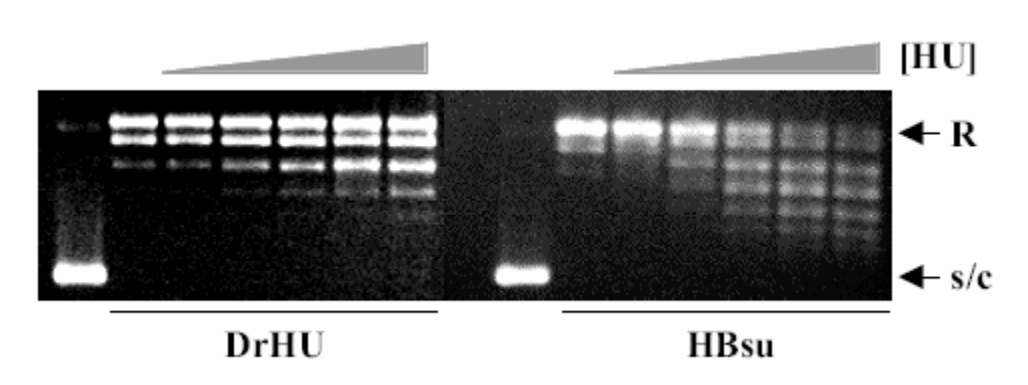

Figure **2.7**. Ability of DrHU to introduce DNA supercoils. Supercoiling of relaxed DNA (R) in the presence of 0 μM, 0.1 μM, 0.25 μM, 0.5 μM, 0.75 μM, 1.0 μM of DrHU (left panel) and HBsu (right panel). Lanes 1 and 8, supercoiled (s/c) pGEM5 DNA.

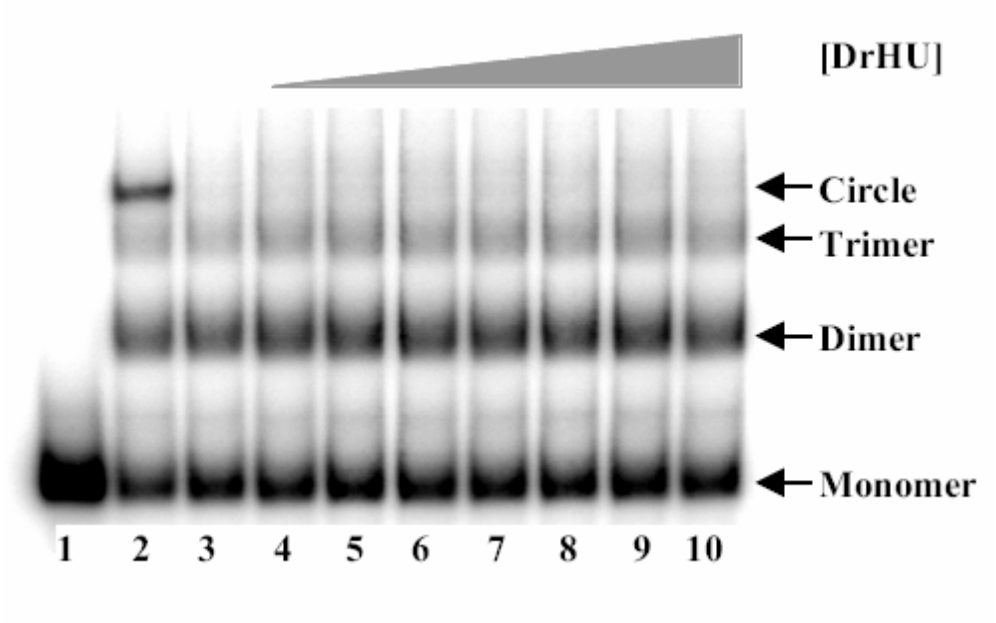

Figure **2.8**. DrHU is unable to circularize 105 bp DNA in the presence of T4 DNA ligase. Lane 1 shows 105 bp linear duplex DNA. Lane 2 shows DNA coincubated with ligase and 200 nM HBsu with formation of circles, as indicated. Lane 3 represents DNA incubated with ligase alone. Lanes 4-10 shows 105 bp linear duplex DNA incubated with ligase and increasing concentrations (12.5, 25, 50, 75, 100, 125, 150 nM) of DrHU. Samples were incubated for 60 min. Circular DNA was confirmed by its resistance to digestion with Exonuclease III.

and an associated preference for DNA with flexure points such as nicks or gaps have been inferred. Our data show that neither of these expectations have been realized for DrHU, which instead exhibits preferred binding only to pre-bent DNA substrates. The implications regarding *in vivo* functions of DrHU and general mechanisms of DNA binding and bending by HU proteins are discussed.

Possible *in vivo* Function of DrHU

Unusual DNA-binding properties are evidenced by the nonspecific site size of almost 50 bp, which was suggested by formation of stable complexes only on DNA of ~50 bp (Figure 2.5A). The possibility remains that complexes are stable to electrophoresis only after multiple protein molecules aggregate on the DNA, however, the binding isotherm is hyperbolic, ruling out significant cooperativity of binding. The unique 47-amino acid N-terminal extension that precedes the sequence characteristic of HU proteins is lysine-rich and predicted to adopt a kinked helix conformation, much like that seen in eukaryotic histone H1, suggesting a role in DNA binding. The potential role of this extension in conferring a longer site size may now be determined by analysis of a mutant protein that is deleted for this unique segment. Another unexpected feature, the inability to bend duplex DNA that is evidenced by the failure of DrHU to promote DNA circularization (Figure 2.8), argues against an architectural role for DrHU in mediating local DNA distortion.

A role for *E. coli* HU in DNA repair has been suggested to correlate with its >100-fold higher affinity for DNA with nicks or gaps compared to duplex DNA [12, 14, 16]. In contrast, DrHU binds only marginally better to DNA with local distortions such as nicks or 4-nt loops (Figures 2.4 and 2.5). This suggests that DrHU is not only unable to bend duplex DNA, but that DNA with increased flexure confers no energetic advantage. Instead, DrHU binds with high

affinity only if the energetic demands are significantly lessened by predisposed DNA bends, as achieved by bulge-loops (Figure 2.4D) or 4-way junction structures (Figure 2.6). An *in vivo* role for DrHU in recognition of damaged DNA is therefore not supported by its *in vitro* DNA binding properties. Rather, the ability of DrHU to bind preferentially only to 4-way junction structures may correlate with an *in vivo* role in DNA recombination events. While the extensive and accurate repair of double-strand breaks characteristic of *D. radiodurans* is expected to include template–independent recombination events, proposed to be facilitated by restricted diffusion of DNA fragments [43], template-dependent recombination is also expected to contribute to efficient double-strand break repair. A functional RecA protein is required for manifestation of the resistance phenotypes in *D. radiodurans* where it has been proposed to play a role in bringing together overlapping DNA fragments [44, 45]. DrHU may serve to stabilize 4-way junction structures in preparation for subsequent repair events.

The Basis for Substrate-selectivity of HU Proteins

Differential substrate selectivity by HU homologs is evidenced by the length of binding sites engaged by members of this protein family. *E. coli* HU was reported to engage only ~9 bp of duplex DNA, while IHF, TF1 and *T. maritima* HU were shown to bind a ~37 bp site [10, 22, 23]. These proteins all share an ability to bend duplex DNA; IHF and TF1 share with *E. coli* HU a marked preference for DNA with local distortions such as nicks and 4-nt loops [7, 9, 10], and *T. maritima* HU mediates DNA circularization and supercoiling *in vitro* (A. Sokunbi and A.G., in preparation). DrHU is unique in its failure to bend duplex DNA and the first member of the type II DNA binding protein family whose binding properties argue against its function in an architectural capacity.

We note that DrHU binds marginally better to DNA containing 4-nt loops, nicks (Figure 2.4B) or hmU-for-T substitutions compared to DNA with gaps (Figure 2.4C). DNA with loops cyclizes efficiently in the presence of DNA ligase, and the cyclization probability (J factor) depends very little on the length of the DNA, suggesting the presence of both torsional and bending flexibility [46]. Recent reports showed that nicks result in a limited decrease in the bending force constant compared to perfect duplex, with a <8° bend towards the opposing strand, however, significant torsional flexibility was detected [47, 48]. In contrast, DNA with gaps was shown to be anisotropically bent, although the measurements did not allow conclusions about torsional flexibility [49]. We surmise that the configuration of the DNA bend imposed by gaps, combined with potentially distinct dynamic properties of the DNA, render a less favorable substrate for DrHU binding compared to duplex with loops or nicks.

DrHU recognizes a structure characteristic of the 4-way junction. Whereas the junctions are expected to exist as an equilibrium mixture of two conformational isomers with the stacked junction arms exhibiting an interduplex angle of 40-60°, the sequence at the crossover affects their ratio [39, 40, 50]. When DrHU forms a single complex, only two strands exhibit partial protection, suggesting that one isomer predominates, and that DrHU preferentially binds one pair of junction arms. The region of protection of strands 2 and 3 distal to the crossover would correspond to interior strands facing each other, indicating that DrHU is positioned between two junction arms, protecting interior strands while exposing the crossover and the opposite strands to enhanced cleavage (Figure 2.6). A second DrHU dimer protects the opposite pair of junction arms sharing a similar structure, again leaving the crossover unprotected. This positioning of two DrHU dimers would not favor protein-protein contact and would therefore be consistent with the observed lack of cooperativity (Figure 2.6B). By comparison, two *E. coli* HU dimers were seen

to protect preferentially strands 2 and 4 and to cause partial protection primarily at the crossover [13]. However, the smaller, heterodimeric *E. coli* HU binds asymmetrically and in an equivalent position to both 4WJ and nicked DNA, generating a single DNA kink of ~65° at the break or junction point by intercalation of Pro63 of the β-arm while the α-arm interacts with the 3'-branch, unable to kink the duplex DNA [15]. DrHU evidently engages the 4WJ differently from *E. coli* HU, with symmetrical contacts to the interior strands of a pair of junction arms that leaves the crossover exposed (Figure 1.2).

The unique properties of DrHU imply that substrate selectivity by HU proteins is dictated by a differential ability of the proteins to stabilize the proline-mediated DNA kinks. Optimal stabilization leads to high affinity and significant bending of duplex DNA and an only modest preference for more flexible DNA structures, as seen for *T. maritima* HU [22]. The ability to distinguish sequence-dependent DNA flexure leads to the stabilization of distinct bend angles in different DNA substrates [8]. For example, limited stabilization of the kinks in duplex DNA leads to a short, ~9 bp nonspecific site size, as seen with *E. coli* HU, which secures an energetic advantage on binding DNA with flexure points such as nicks and gaps [16]. For DrHU, the stabilization of DNA kinks that is required for DNA bending can not be achieved even in DNA with imposed flexure. Only DNA with predisposed bends sufficiently lessen the energy required for bending, causing high affinity binding by allowing DNA distal to the bend to come into protein contact. As a consequence of a differential ability to stabilize the proline-mediated DNA kinks, HU proteins should therefore be expected to exhibit distinct substrate preferences and associated *in vivo* roles.

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CHAPTER 3

THE N-TERMINAL EXTENSION OF *DEINOCOCCUS RADIODURANS* HU CONTRIBUTES TO DNA BINDING AND CONFERS UNIQUE PLACEMENT ON FOUR-WAY JUNCTION DNA

Introduction

HU proteins are ubiquitous in prokaryotes, and they are abundant [1-3]. These dimeric proteins are structurally conserved, with α-helices coming together to form a compact "body", from which β-sheets extend to form "arms" that encircle the DNA [4-6]. The structures of integration host factor (IHF) from *Escherichia coli* and *Anabaena* HU in complex with DNA show that two prolines at position 63 intercalate into the DNA base-pair stack, forming two kinks in the DNA. While an ~160° bend is induced by IHF, *Anabaena* HU introduces a variable bend of 105-140° in the DNA [7, 8].

In *E. coli*, where it has been most widely studied, HU functions primarily as an architectural protein. It binds double-stranded DNA non-specifically and with low affinity and with considerably higher affinity (nM) for distorted DNA, causing significant DNA bending and negative supercoiling [9-13]. Studies indicate that it compacts the DNA into a nucleosome-like structure, [14] and that it plays a role in transcriptional regulation [15], Mu transposition [16] and DNA replication [17-19]. Cells lacking HU are highly sensitive to γ and UV irradiation, suggesting its role in DNA repair and recombination [18-21].

Deinococcus radiodurans is a mesophilic eubacterium with a remarkable capacity to survive the effects of double strand DNA breaks incurred upon exposure to ionizing radiation or prolonged dessication [22, 23]. Upon exposure to environmental stress, it can reconstitute its genomic DNA from 1000 to 2000 double strand breaks, whereas an excess of 10-15 double

strand breaks is lethal to *E. coli* [24]. The annotated genomic DNA sequence has left few clues to the unique characteristics of this organism [25], however, since it has multiple genome equivalents, it has been suggested that efficient homologous recombination may be a contributing factor to the resistance [26-28].

We recently reported that *D. radiodurans* HU (DrHU) has unique DNA-binding properties [29]. While DNA-binding site sizes for HU homologs vary, from ~37 bp for *E. coli* IHF and *Thermotoga maritima* HU [7, 30], to an optimal binding site of ~19 bp for *Anabaena* [8] and *Helicobacter pylori* HU [31] and a much shorter DNA binding site of ~9 bp for *E. coli* HU [13, 32], DrHU has an optimal binding site of ~50 bp, which is the largest reported for any protein of this family. DrHU also has a distinct preference for four-way junctions compared to linear double stranded DNA: the protein binds to the outer arms of the two strands of the junction, while leaving the crossover exposed for enhanced cleavage. This is in contrast to *E. coli* HU, which was shown to protect primarily the junction crossover [10, 12].

DrHU has an extra 47 amino acids at the N-terminus, which is a unique feature, followed by the conserved DNA-binding fold. We show here that a truncated DrHU (ΔDrHU) that has only the DNA binding fold binds to double stranded DNA with a binding site size of ~17 bp. While ΔDrHU also binds preferentially to four-way junction DNA, the absence of the N-terminus changes the mode of binding to the DNA with ΔDrHU protecting mainly the junction crossover. Our data also suggest that four-way junction DNA assembles a complex composed of DrHU as well as other *D. radiodurans* encoded protein(s). These data suggest that the N-terminal extension of DrHU serves to place the protein uniquely on four-way junction DNA and that DrHU may be playing a regulatory role in DNA recombination.

Materials and Methods

Cloning, Overexpression and Purification of ΔDrHU

The gene encoding ΔDrHU was amplified from plasmid pET-DrHU [29] with primers ΔDrHU–up (5'-GCTCTATCCCCATATGCCCCCCTTCACAGC-3') encoding a methionine at the 48th amino acid instead of lysine and a reverse primer ΔDrHU-down (5'-CGGAGGGAGCGGTCACATATGAACCCGCTTACAGG-3') which was positioned at the start site of the DrHU gene and reading into the plasmid pET5a. The PCR product with *Nde I* sites at both ends (as indicated with underlines) was digested and ligated with T4 DNA ligase to form the plasmid pET5a-ΔDrHU. The integrity of the plasmid was confirmed by sequencing. The plasmid was transformed into *E.coli* BL21(DE3)pLysS and induced for overexpression with 1mM IPTG at OD_{600}=0.3. Cells were harvested two hours after induction and stored at -80^{o}C.

The purification of ΔDrHU was carried out at 0-4^{o}C. Cells were resuspended in lysis buffer [29], and lysed by sonication. The cell lysate was fractionated by ammonium sulfate precipitation, as described [33]. The precipitate that formed with 75% $(NH_4)_2SO_4$ was collected, dissolved in buffer A (20 mM Tris-HCl (pH7.0), 50 mM KCl, 5% glycerol, 1 mM Na_2EDTA, 3.5 mM 2-mercaptoethanol, and 0.2 mM phenyl methyl sulfonyl fluoride (PMSF)), dialyzed against buffer A, and applied to a preequilibrated CM sepharose column. The protein was eluted with a linear gradient from 50mM to 1M KCl in buffer A. Peak fractions were adjusted to 50% saturation with $(NH_4)_2SO_4$ and loaded on a phenyl sepharose column equilibrated in buffer A containing 50% $(NH_4)_2SO_4$. ΔDrHU was eluted with a linear gradient from 50% to 0% $(NH_4)_2SO_4$. Peak fractions were pooled and dialyzed against buffer A and loaded on a hydroxyapatite column equilibrated with buffer A, and eluted as described for the CM-sepharose column. Peak fractions were pooled and dialyzed in buffer A and applied to a heparin-sepharose

column at pH 7.0 and eluted as described for the CM-sepharose column. Purity was ascertained by Coomassie staining of gels overloaded with protein. Protein concentrations were determined by Coomassie blue stained SDS-polyacrylamide gels, using BSA as a standard. Gluaraldehyde-mediated cross-linking of ΔDrHU was performed as described [29].

Western Blotting

DrHU (5-10 ng) and ΔDrHU (5-20 ng) were electrophoresed on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane at 100V for 2 hrs. The membrane was blocked for non-specific binding using 5% nonfat milk in 1x Phosphate Buffered Saline with 0.1% Tween-20 (PBST) for 1hr. The membrane was incubated for 1hr with mouse monoclonal anti-histone H1 antibody (mAb 3A10, [34]), generously provided by P. DiMario, followed by incubation with secondary antibody anti-mouse-IgG for 1hr and incubation with a colorimetric substrate Opti-4CN (Biorad). Each step was preceded by two short washes in 1xPBST buffer. The mouse histone H1 antibody was purified from culture supernatants using protein G (Sigma). The supernatant was incubated with protein G-agarose beads in 20mM sodium phosphate buffer, pH 7.0, washed and eluted with 100 mM glycine HCl, pH 2.7. Equal amounts of 1M Tris, pH 8.0 was added to raise the pH of the antibody, which was stored at -80^{o}C.

D. radiodurans cell lysates were prepared from cultures grown at 30^{o}C for 48 hours. Cell pellets were stored at -80^{o}C, resuspended in buffer A, sonicated, and the lysate centrifuged at 15,000g for 20 minutes. The supernatant was dialyzed against buffer A and aliquots stored at -80^{o}C.

Circular Dichroism Spectroscopy

Circular Dichroism (CD) spectra were recorded as described [31]. The protein concentration was 0.1 mg/ml in 10 mM sodium phosphate buffer pH 7.0 with 50 mM NaCl for

wavelength scans. For the thermal denaturation, spectra were recorded from 190-250 nm with 1 nm increments at each temperature with 0.05 mg/ml of protein in the same buffer. Data was collected from 5-90˚C in 5˚ intervals. CD signals at 218, 219, 220 and 221 nm was used for analyses of thermal denaturation curves which were fit to a modified van't Hoff equation [35].

$$\Delta\theta = [(m_n*T + b_n) + (m_d*T + b_d)]*K/(1+K) \text{ and } K = \exp((-\Delta H^o(1-T/Tm))/RT)$$

Where $\Delta\theta$ is the molar ellipticity, m_n and b_n are the slope and intercept of the native state, m_d and b_d are the slope and intercept of the denatured state, T is the temperature in degree Kelvin, Tm is the melting temperature (°K), ΔH^o is the van't Hoff enthalpy and R is the gas constant. Data were fitted using KaleidaGraph.

Electrophoretic Mobility Shift Assays

EMSA was performed using 8% (w/v) polyacrylamide gels (39:1(w/w) acrylamide:bisacrylamide) in TBE (45 mM Tris-borate pH 8.3, 1mM EDTA) [29]. Reaction conditions were as described [29], and each sample contained 100 fmol DNA in a total reaction volume of 10 µl, unless otherwise mentioned.

Binding isotherms were analyzed using non-linear fits of DNA titrated with protein, using the equation $Y= (Y_{max}X)/(K_d + X)$ where K_d is the observed equilibrium dissociation constant for protein binding to a single site, Y is fractional saturation and X is protein concentration. The nonspecific binding site size was calculated from titrations under stoichiometric conditions.

EMSA with four-way junction (4WJ) DNA was performed as described [29]. Ten fmoles of DNA was incubated with 9 µl of cell lysate or 80 nM DrHU in the presence of antibody (5 µl) for 5 minutes and terminated by loading onto a prerun 8% polyacrylamide gel. The gel was run at 200 V for 3 hours, dried, exposed overnight and quantitated by phosphorimaging using

Imagequant 5.0 software. Cell lysates were incubated with DNA and increasing amounts of DrHU under the same conditions. Supershift assays were performed by assembling reactions containing cell lysate and/or DrHU as well as antihistone H1 antibody. Equal amounts of cell lysate were electrophoresed on 10% SDS-PAGE gels, and western blotting was performed with antihistone H1 as a primary antibody as described above.

Two-dimensional Methidium Propyl EDTA (MPE)-Fe(II) Footprinting

Two-dimensional footprinting was perfomed essentially as described [29]. Each reaction contained 0.4 pmoles of DNA. DNA cleavage was performed with 1 μl of 10 mM sodium ascorbate and 2μl of freshly prepared 25 μM MPE-Fe(II) for two minutes, and reactions were terminated by loading on a 8% native polyacrylamide gel. Protein-DNA complexes were isolated and resolved on a 15 % denaturing gel. Density profiles were obtained by phosphorimaging.

Supercoiling Assays

Negatively supercoiled pGEM5 (100 ng/reaction) was relaxed with Vaccinia DNA topoisomerase I [29]. Relaxed DNA was incubated for 60 minutes at 37°C with increasing amounts of protein, followed by termination with proteinase K with further incubation at 37°C for 60 minutes. Reactions were resolved on a 1% agarose gel run at 25 V for 14 hours.

DNA Circularization Assay

DNA was prepared from pET5a as described [29]. Time course ligation experiments with increasing protein concentrations were carried out for cyclization studies. Reactions containing 100 fmol DNA and the desired concentration of protein were incubated in 1X binding buffer, 1 X ligase buffer (New England Biolabs) and 0.5 mM ATP in the presence of 80 U of T4 DNA ligase at room temperature for the desired time. Reactions were terminated with proteinase K

followed by incubation for 15 minutes at 55°C, analyzed on 8% native polyacrylamide gels and quantitated by phosphorimaging.

Results

N-terminally Truncated DrHU is Folded in Solution

While most HU proteins consist of the 90-99 amino acid type II DNA binding fold, DrHU has an additional 47 amino acids at its N-terminus, followed by the motif characteristic of type II DNA-binding proteins. The sequence of the N-terminal extension is rich in lysine and highly repetitive, and its similarity to the C-terminus of eukaryotic histone H1, known to have a direct role in DNA interactions [36, 37], predicts its participation in DNA interactions. To specify the function of the DrHU N-terminus, a truncated protein, ΔDrHU was prepared in which the 47 N-terminal amino acids are deleted. ΔDrHU was purified to ~95% homogeneity (Figure 3.1).

Incubation of increasing concentrations of ΔDrHU with 0.1% glutaraldehyde shows that the protein forms similar multimeric assemblies as seen with other HU homologs [31, 38], consisting of dimers, trimers and other higher order structures, in comparison to the predominant dimer form observed with DrHU (Figure 3.1). The formation of multimeric assemblies decreases upon incubation at 0˚C with ΔDrHU, but incubation at 0°C has no effect on cross-linking of the full-length protein (data not shown). Circular dichroism studies show that the far UV spectrum of ΔDrHU has a secondary structure characteristic of HU homologs with significant α-helical content, with a low negative value around 220 nm and 208 nm and a positive value at ~200nm [39] (Figure 3.2A). These results are consistent with those of other HU proteins and suggest that ΔDrHU adopts a folded conformation in solution [40, 41]. Glycine at position 15 has been shown to confer thermostability to HU from thermophilic organisms (e.g. *Bacillus stearothermophilus*

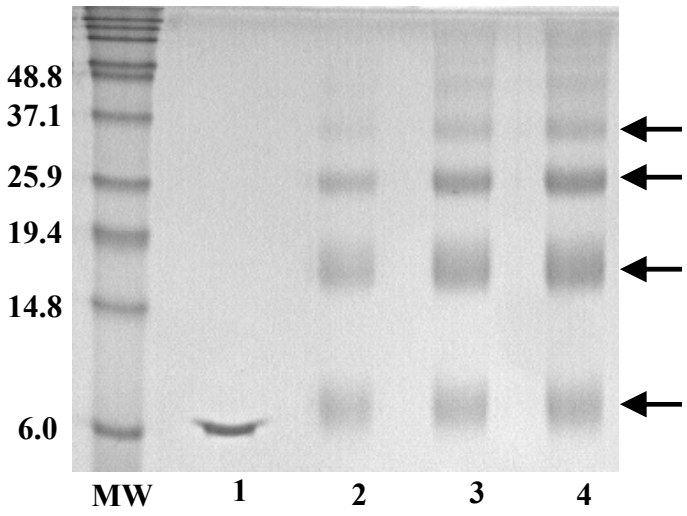


Figure **3.1**. ΔDrHU exists as an oligomer in solution. Lane 1 contains 1 μg of unmodified protein; lanes 2-4, contain 3, 5 and 7 μg of ΔDrHU cross-linked with glutaraldehyde. Molecular weight markers are in kDa. Arrows indicate ΔDrHU in different multimeric forms.

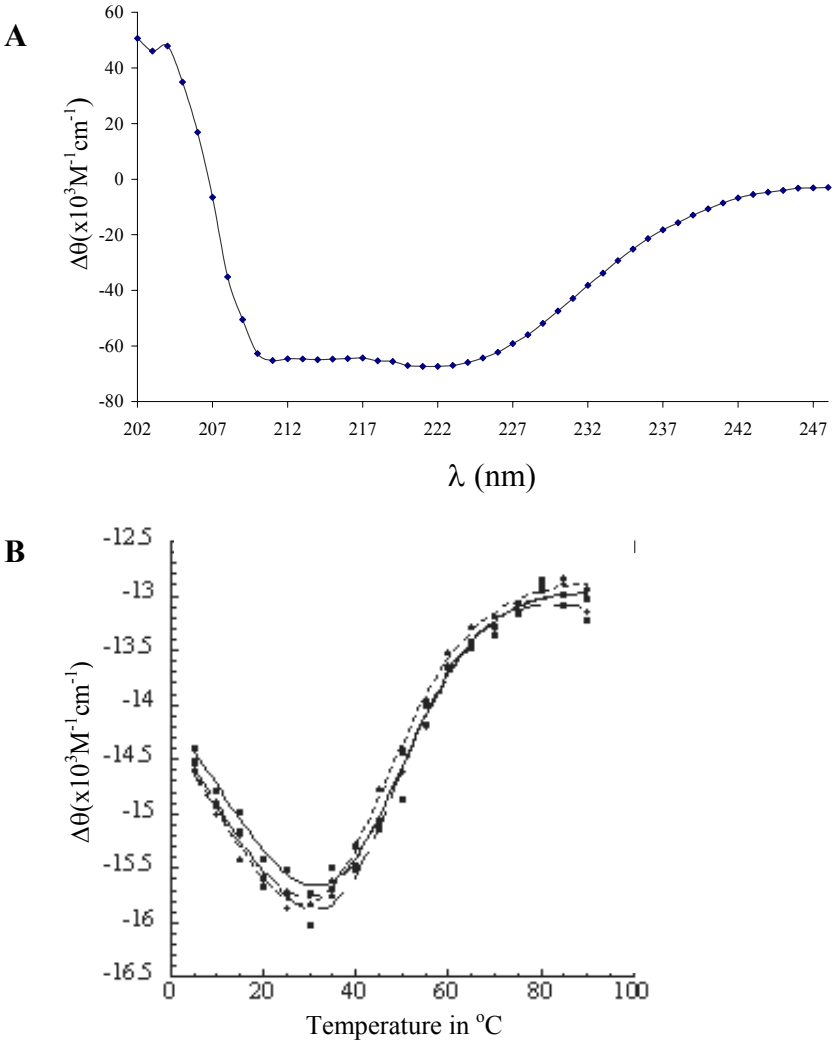


Figure **3.2**. Circular dichroism analysis of ΔDrHU. (A) Far UV spectrum of ΔDrHU reflecting significant α-helical content. (B). Melting curve of ΔDrHU. The thermal denaturation was recorded from 5° to 90° for wavelengths 218 (●), 219 (■), 220 (◆) and 221 (▲) nm, showing a Tm of 46.4±0.1°C.

and *Thermotoga*), where substitution of G→E decreases the melting temperature (Tm) by 11°C [40-43]. Denaturation of ΔDrHU from 5°C to 90°C yields a melting temperature of 46.4±0.1°C (Figure 3.2B) with a slow transition from the folded to the unfolded state. Though ΔDrHU has the G15 residue, the Tm is similar to HU from other mesophilic organisms for example, *B. subtilis* HU whose Tm is 48.6°C in sodium phosphate buffer pH 7.0 containing 200 mM NaCl [38]. We also note the marked increase in ellipticity at ~220 nm on reducing the temperature from ~30°C to 5°C, indicating conformational changes associated with lower temperatures. This is consistent with the reduced cross-linking efficiency of ΔDrHU at 0°C.

Binding Site Size of ΔDrHU

To analyze DNA binding by ΔDrHU, a 37 bp duplex DNA was used. While DrHU is unable to form stable complex with the 37 bp duplex DNA [29], ΔDrHU forms a complex under identical conditions with no indication of cooperativity and a half-maximal saturation of 22.1±1.4 nM (Figure 3.3A), suggesting a smaller binding site size than the ~50 bp site observed with DrHU. To calculate the binding site size, a 50 duplex DNA was used which was generated by nearly equal extension of either end of the 37 bp duplex [29]. Under stoichiometric conditions, where [DNA]> K_d (Figure 3.4A), at 50 mM NaCl, an occluded binding site size for ΔDrHU of 17±1 bp is measured (Figure 3.4B). Half-maximal saturation of 11.9±1.1 nM with no evidence of cooperativity is observed for 50 bp DNA (Figure 3.4C). Thus removal of the N-terminus from DrHU allows the protein to form a stable complex with the 37 bp duplex DNA with a binding site size consistent with other HU homologs, such as *H. pylori* and *Anabaena* HU [8, 31], but still in definite contrast to *E. coli* HU whose binding site is reported to be ~9 bp [10]. The reduced site size measured for ΔDrHU is consistent with a contribution of the N-terminus to DNA binding.

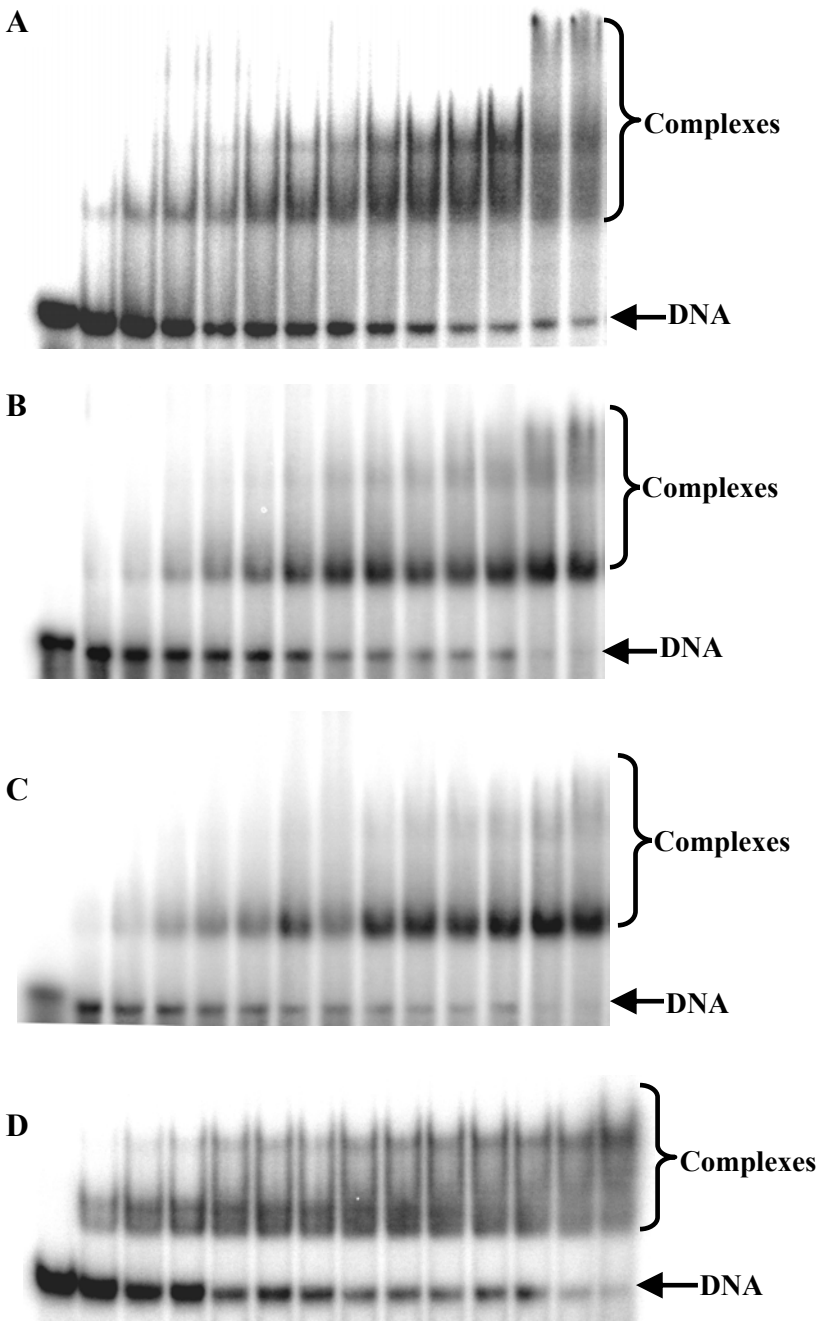


Figure **3.3**. ΔDrHU binds preferentially to pre-bent DNA. Electrophoretic analysis of ΔDrHU with (A), 37 bp duplex DNA; (B), nicked DNA; (C), 1 nt-gap DNA; (D), DNA with bulge. Complexes are indicated at the right; ΔDrHU concentrations, identical for panels (A)-(C) are 0-1.0 μM; for panel (D), 0-100 nM.

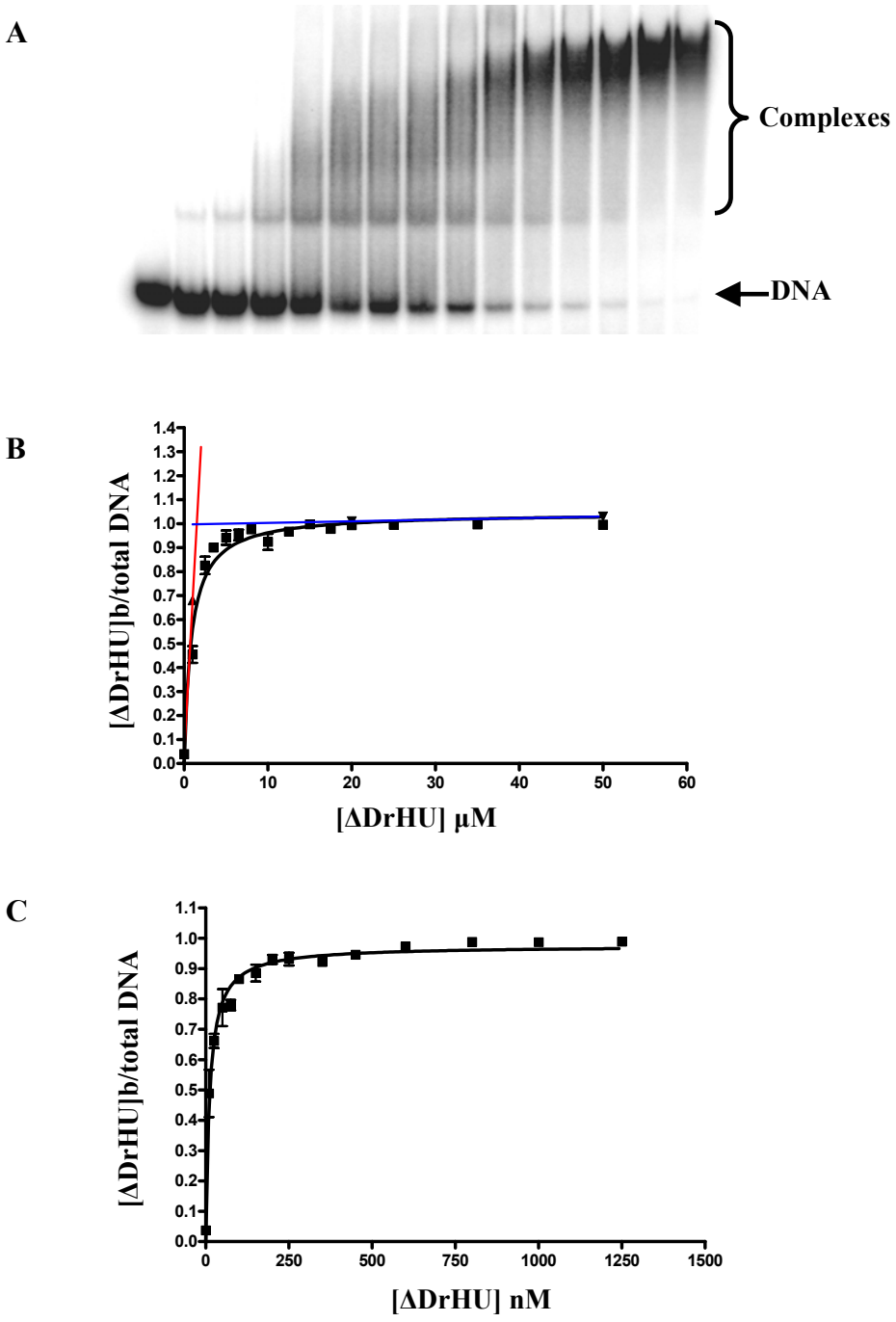


Figure **3.4**. Binding of ΔDrHU to 50 bp DNA. (A), Electrophoretic analysis of 50 bp duplex DNA titrated with ΔDrHU under stoichiometric conditions. Complexes are indicated on the right. Each reaction contains 0.5 µM DNA ([DNA] > K_d) with increasing concentrations of 0- 50 µM of protein. (B), Ratio of bound ΔDrHU to total 50 bp DNA as a function of [ΔDrHU]. (C). Plot indicating the half-maximal saturation of 50 bp DNA in presence of ΔDrHU.

Architectural Properties of ΔDrHU in DNA Binding

Comparison of DNA binding affinities of the full-length DrHU for modified DNA shows that it has little preference for flexible DNA structures such as DNA with loops or nicks [29]. EMSAs with ΔDrHU under identical conditions shows that the protein binds 37 bp duplex DNA with 4-nucleotide (nt) loops, nick, 1-nt gap, 2-nt gap or 3' overhangs (Figure 3.3B-C and data not shown) with no increase in affinity compared to perfect duplex DNA. Incubation of ΔDrHU with 37 bp duplex DNA with a G+C content of 67%, which is similar to the G+C-content of *D. radiodurans* genomic DNA, shows that the protein exhibits no preference over 37 bp duplex DNA with average G+C-content (data not shown). In contrast, ΔDrHU binds DNA in which two loops of three C's have been introduced at a distance of 9 bp, with modestly increased preference compared to perfect duplex DNA, forming multiple complexes, with a half maximal saturation of 9.0±0.8 nM (Figure 3.3D).

Full-length DrHU can introduce negative supercoils into relaxed DNA to a limited extent [29]. However, incubation of relaxed pGEM5 DNA with ΔDrHU is unable to introduce supercoils into relaxed plasmid DNA (Figure 3.5). Thus the limited ability of DrHU to form supercoils is abolished with the removal of the N-terminus, suggesting that formation of a toroidal superhelix requires the N-terminal extension.

To assess the DNA bending ability of ΔDrHU, a circularization assay was used in which DNA shorter than the persistence length is circularized with T4 DNA ligase. Like wild-type DrHU, ΔDrHU is also unable to promote cyclization of 105 bp duplex DNA or 105 bp duplex DNA with nick in the center (data not shown). Evidently, both the full-length and the truncated protein are unable to circularize DNA, otherwise a general property of other architectural proteins.

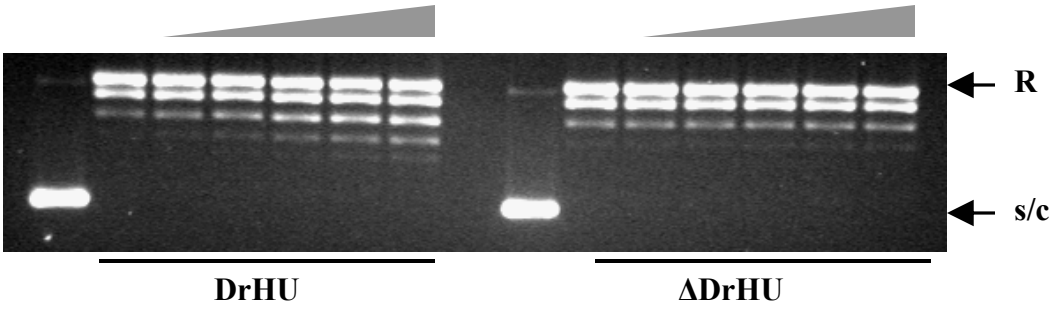


Figure **3.5**. Inability of ΔDrHU to introduce DNA supercoils. Supercoiling of relaxed DNA (R) in the presence of 0 μM, 0.1 μM, 0.25 μM, 0.5 μM, 0.75 μM, 1.0 μM of DrHU (left panel) and ΔDrHU (right panel). Lanes 1 and 8, supercoiled (s/c) pGEM5 DNA.

DrHU and ΔDrHU Interact Differently with Four-way Junction DNA

HU proteins have been shown to participate in DNA inversion, recombination, and repair, reflected in an increased affinity of *E.coli* HU for four-way junction (4WJ) structures. A 4WJ DNA was made as reported for analysis of *E.coli* HU [10]. With ΔDrHU, a ladder of complexes forms with increasing protein concentrations (Figue 3.6A). No evidence of cooperativity is observed, and the half maximal saturation of the binding isotherm is 2.3±0.3 nM (Figure 3.6B). Linear duplex DNA corresponding to the two arms of the four-way junction, a 35-mer and a 40-mer respectively, showed the same binding pattern as seen with 37 bp duplex DNA (data not shown). This is in contrast to full-length DrHU which forms two complexes on four-way junction DNA [29] and consistent with a shorter site size for ΔDrHU.

The position of ΔDrHU on the four-way junction was assessed by two-dimensional MPE-Fe(II) footprinting, measuring ΔDrHU binding to the stacked X conformation preferred in the presence of Mg^{2+}. We assayed binding of ΔDrHU in complexes 1 and 2, the two complexes of densitometric traces upstream of the crossover in strand 1 beyond which the counts were too weak to be accounted for. For strand 2, ΔDrHU in complex 1 binds to the region of the highest mobility, to compare with the full-length protein. In complex 1, ΔDrHU protects the four strands of the 4WJ differently (Figure 3.6 C-F). It shows insignificant change in the densitometric traces upstream of the crossover in strand 1 beyond which the counts were too weak to be accounted for. For strand 2, ΔDrHU in complex 1 binds to the region of the crossover, and in strand 3, it binds mainly to the region round the crossover and downstream of it. The densitometric profiles corresponding to complex 2 shows protection of strands 2 and 3 in the region of the crossover with an interduplex angle of 40-60°. With one conformational isomer, ΔDrHU binds to the crossover region of strands 1 and 3. With the second isomer, the protein binds to the crossover

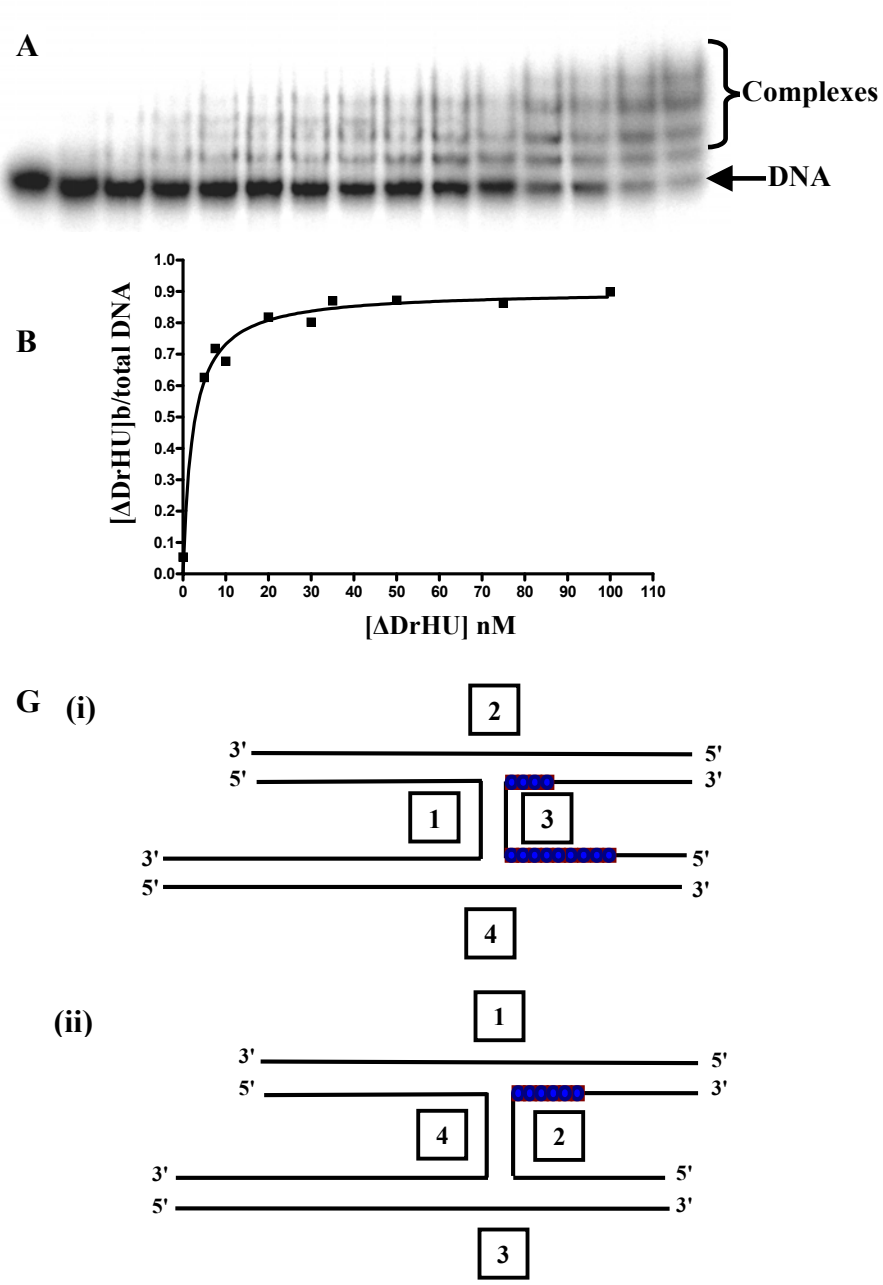
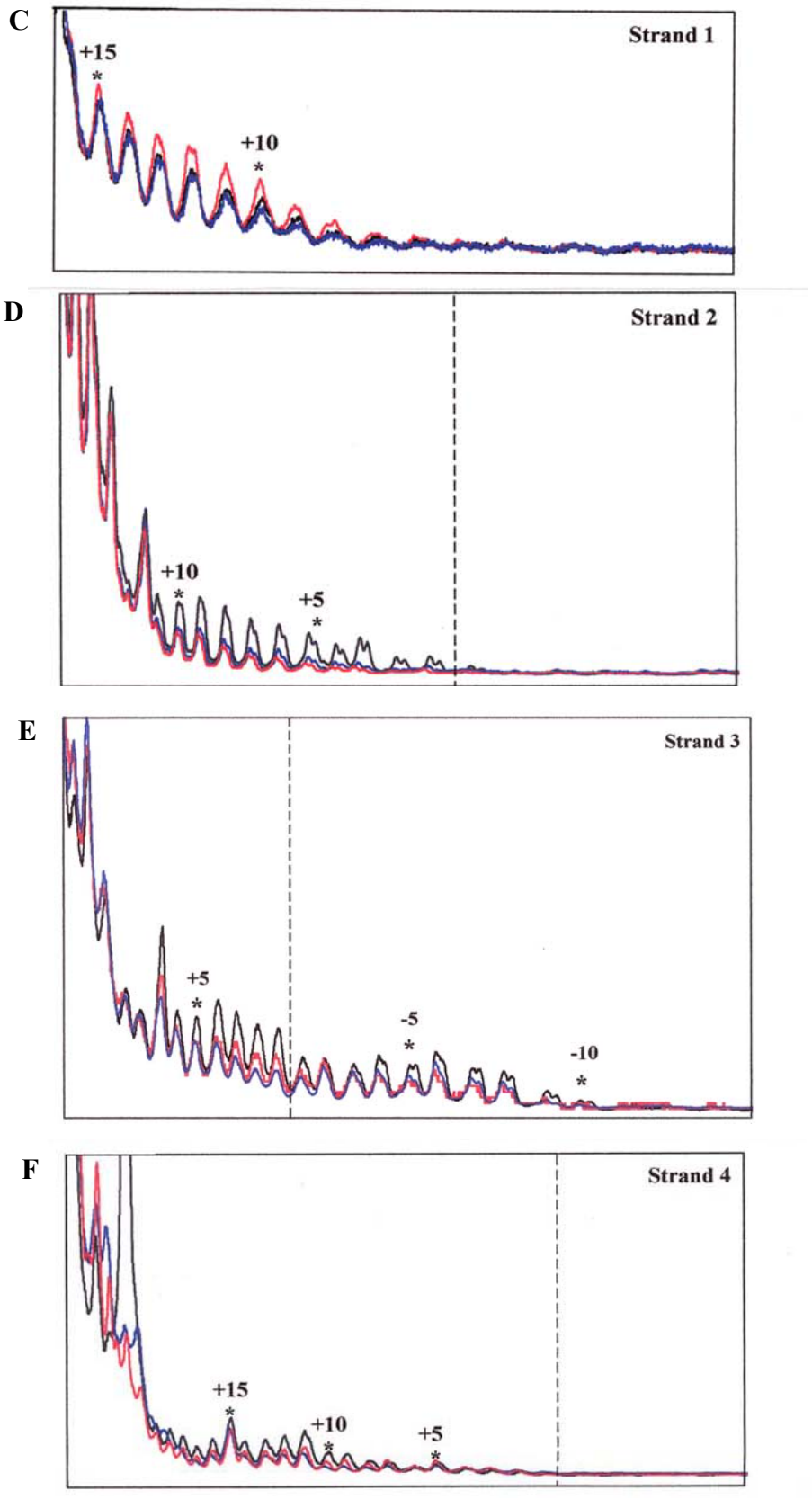


Figure **3.6.** Binding of ΔDrHU to four-way junction. (A). Electrophoretic analysis of ΔDrHU with increasing concentrations of ΔDrHU (0-100 nM). Each reaction contained 1.0 nM DNA. Complexes and free DNA are indicated on the right. (B). Binding isotherm for ΔDrHU binding to 4WJ DNA. Densitometric profiles of (C), strand 1; (D), strand 2; (E), strand 3; (F), strand 4, cleaved in the presence of MPE-Fe(II) in the ΔDrHU-4WJ complex. Black lines indicate DNA only, blue lines indicate ΔDrHU-DNA complex 1 and red lines indicate ΔDrHU-DNA complex 2. Vertical dotted lines indicate the crossover, and positive and negative numbers identify positions downstream and upstream of the crossover, respectively. (G). Summary of cleavage pattern with (■) representing protection by complex 1 and 2; with (i) showing the position of protein with DNA in conformation 1, and (ii) in conformation 2. (figure continued)


C
Strand 1
+15
*
+10
*
D
Strand 2
+10
*
+5
*
E
Strand 3
+5
*
-5
*
-10
*
F
Strand 4
+15
*
+10
*
+5
*

(Figure 3.6D-E). Strand 1 and 4 shows no protection or enhancement by complex 2. The four-way junctions are expected to exist as an equilibrium mixture of two conformational isomers region of strand 2 in complex 1 and 2 and we would expect to see a protection of strand 4 across the junction. The pattern of protection by complex 1 and 2 for strand 4 is not consistent. As these were data collected on different days, it may be that ΔDrHU preferred binding to conformation 1 thus protecting strands 1 and 3, in which case we do not see any protection of strand 2 and 4. This is consistent with the lack of protection observed for strand 4 and we require further data from this strand to confirm this model of binding by ΔDrHU. Based on the present data ΔDrHU binds to the junction crossover for strands 2 and 3, whereas for DrHU it binds alternately to the outer arms of strands 1 and 4 or strands 2 and 3 and allowing the crossover for enhanced cleavage.

The N-terminus of DrHU is Recognized by Histone H1 Antibodies

Eukaryotic histone H1 protein binds DNA, with the C-terminal domain primarily associated with binding to chromatin [36, 37]. Sequence alignments show that part of the 47 amino acid N-terminal domain of DrHU bears significant sequence similarity to the (S/T)PKK motifs found in the C-terminal domain of histone H1; DrHU contains three APA(A/K)K repeats. Probing DrHU and ΔDrHU with antibodies against histone H1 by Western Blotting shows that DrHU is recognized by antibodies to anti Histone H1, whereas ΔDrHU missing the N-terminus is not (Figure 3.7A, B), suggesting that the lysine-containing repeats in DrHU mimic those of H1. Since the coomassie stained gel shows that proteins are left after transfer, it could be a possibility that the failure of ΔDrHU to react with the antibody is due to failed transfer.

The only protein from *D. radiodurans* cell lysate that is recognized by the anti-H1 antibody migrates equidistant with the purified DrHU (Figure 3.7A). We therefore explored the possibility

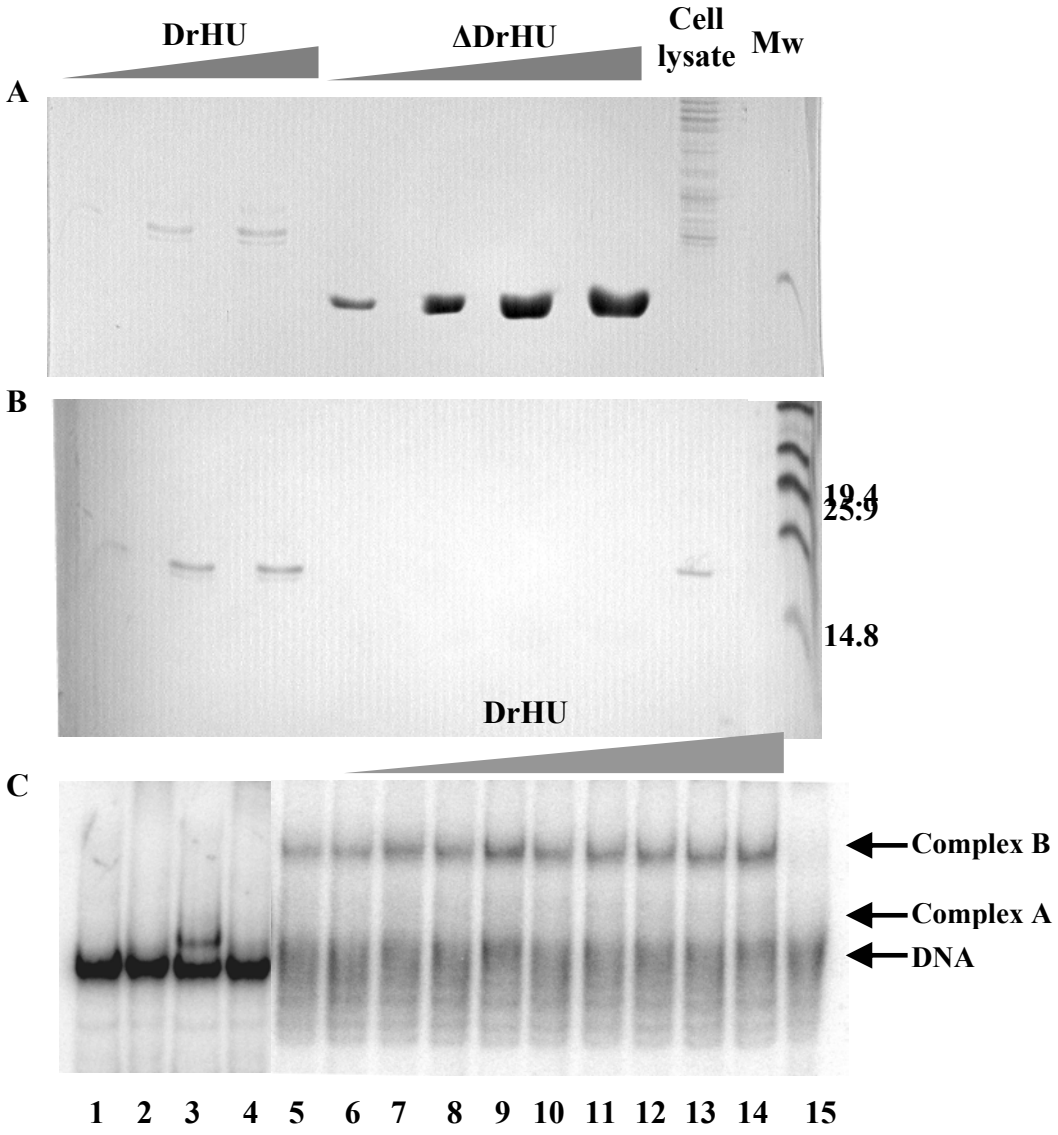


Figure **3.7**. Interaction of DrHU with histone H1 antibodies. (A), SDS-PAGE gel with increasing concentrations of DrHU (lanes 1-3), ΔDrHU (lanes 4-7) and cell lysate (lane 8) stained with Coomassie after transfer. Molecular weight marker is in lane 9. B, Western blotting of gel shown in A using antibody against mouse histone H1. C, EMSA showing incubation of 4WJ DNA with DrHU, with DrHU-DNA complex identified as complex A; and coincubation of cell lysate with increasing amounts of DrHU as complex B. Lane 1, DNA only; lane 2, DNA with antibody (5 µl); lane 3, DNA with DrHU (0.8 pmoles); lane 4, DNA with DrHU (0.8 pmoles) and antibody (5 µl); lane 5, DNA with cell lysate (9 µl); lanes 6-14, DNA with cell lysate and increasing amounts of DrHU up to 25 pmoles; lane 15, DNA with cell lysate, DrHU (25 pmoles) and antibody (5 µl). The DNA degradation observed in lanes 5-15 in presence of cell lysate and DNA is due to the presence of nucleases.

that DrHU might be present in sufficient quantity in *D. radiodurans* lysates to be detected via its preferred binding to 4WJ DNA. The complex that was formed in the presence of DrHU or cell lysate, respectively, upon incubation with the 4WJ was completely disrupted in the presence of the antibody, which indicates its interaction with a protein in the complex. Upon incubation of the cell lysate with the 4WJ DNA, a complex was formed which migrated much slower than the DrHU-DNA complex. Addition of increasing amounts of DrHU to the cell lysate-DNA mixture resulted in enhanced formation of the slow-migrating complex only (Figure 3.7C), which suggests that it may be due to interaction of DrHU with another protein that also recognizes the 4WJ. Attempts were made to study the interaction of the cell lysate directly with the 4WJ using biotinylated DNA immobilized on streptavidin beads, but DrHU was found to bind with high affinity to the streptavidin (not shown).

Discussion

Sequence and Structural Considerations

Comparison of the sequence of DrHU with that of other HU homologs reveals the conservation of the type II DNA-binding fold except that the Gly-Arg-Asn-Pro (GRNP) sequence motif containing the DNA-intercalating Proline at the tip of the β-arms is replaced with GVRP. In *Helicobacter pylori* HU, (HpyHU), GRNP is replaced with GKVP. Restoring the GRNP sequence generates an HpyHU mutant protein with reduced preference for flexible DNA [31], which suggests that subtle changes in this conserved region may result in significant changes in DNA binding. It is conceivable, therefore, that the GVRP sequence of *D. radiodurans* is contributing to the lack of preference for flexible DNA.

DrHU has a Glycine in the loop between helices 1 and 2, otherwise associated with HU from thermophiles, where it causes increased thermal stability. However, ΔDrHU shows no such

enhanced stability. The presence of Gly 15 confers a flexibility to the loop connecting helices 1 and 2 that correlates with optimal helix packing and thermal stability as seen in *B. stearothermophilus* HU, where substitution of Gly15 with Glu reduces loop flexibility and leads to a reduction in Tm from 64˚C to 54˚C. While mutation of Glu15 to Gly increases loop flexibility in *B. subtilis* HU (HBsu) and in the *B. subtilis* bacteriophage SPO1-encoded HU homolog, TF1, it increases the Tm by 11˚C [38, 40-43]. Based on the Tm reported for HBsu of 48.6˚C measured by CD spectroscopy in phosphate buffer pH 7.0 with 200 mM NaCl by Kawamura and co-workers (1996), the Tm of 46.4˚C measured for ΔDrHU under somewhat less stringent conditions (phosphate buffer pH 7.0 with 50 mM NaCl; Figure 3.2) shows comparable thermal stability. However, DrHU also does not conserve the GFG motif at the dimer interface, but substitutes the F for L. This substitution in the hydrophobic core of the protein may therefore also affect thermal stability. Whether absence of the N-terminal tail affects thermal stability or the cold-sensitive behavior of ΔDrHU awaits determination. The incomplete cross-linking observed for DrHU and ΔDrHU is also seen for HBsu where significant monomer is present after cross-linking (unpublished). ΔDrHU exists as dimer (Figure 3.1) suggesting an essentially unaltered dimer interface compared to other HU proteins, and the secondary structure of ΔDrHU based on the far-UV CD spectrum shows a backbone polypeptide conformation similar to that of other HU homologs. These observations suggest that the N-terminus constitutes a separate domain of DrHU.

The N-terminal tail of *D. radiodurans* HU contains three APA(A/K)K repeats, reminiscent of the (S/T)PKK repeats found in the C-terminus of eukaryotic histone H1. The C-terminal tail is disordered in aqueous solution, but becomes ordered on DNA binding or in trifluoroethanol [37, 44]. The similarity of the repeat sequences suggests that the N-terminus of

DrHU may also be unfolded in aqueous solution due to charge repulsion. The (S/T)PKK motif-containing region may adopt a helical conformation similar to that characteristic of High Mobility Group (HMG) box proteins where the clustered lysines bind DNA through neutralization of phosphates and facilitate DNA condensation [37, 38]. These considerations suggest that the N-terminal domain of DrHU would also participate in DNA binding.

Substrate Selectivity of ΔDrHU

The DNA-binding fold of DrHU (ΔDrHU) has a non-specific DNA binding site size of ~17bp. This is similar to the binding site size seen for HU homologs from other mesophilic organisms, namely *Anabaena* and *H. pylori* HU. The unusual DNA-binding properties exhibited by DrHU with a non-specific site size of almost 50 bp, suggested by the formation of stable DNA-protein complexes only on DNA ~ 50 bp, implies that the enhanced site size is due to the positioning of the N-terminal tail on the DNA. The lack of supercoiling by ΔDrHU also constitutes evidence that the N-terminus contacts DNA, and it is consistent with the contribution of the H1-like repeats in DNA compaction [37]. Since ΔDrHU does bind DNA, it must either be unable to wrap DNA in toroidal supercoils or the path of wrapping is not consistent with the introduction of supercoils. For example, the HU homolog IHF binds negatively supercoiled DNA and wraps the DNA that lies in nearly a single plane with a small dihedral angle ~10˚-15˚ and with an average helical twist of 33.3° similar to that of B-form DNA [7], while HU from *Anabaena* introduces a larger dihedral angle that leads to DNA supercoiling [23].

ΔDrHU has no increased preference for distorted DNA, like loops, nicks, gaps or overhangs. However, like DrHU it exhibits higher affinity for DNA with bulges (half-maximal saturation of ~9 nM) and for four-way junction structures (half-maximal saturation of ~2.3 nM), indicating it prefers binding to DNA where the energetic cost of bending has been reduced. The

four-way junctions are expected to exist in an equilibrium mixture of two conformational isomers with the stacked junction arms having an interduplex angle of 40-60°, with the sequence of the crossover affecting their ratio [46-48]. When DrHU binds to four-way junction DNA forming complex 1 at low concentrations, it binds to the outer arms of strands 2 and 3 thus protecting the interior of the strands facing each other, leaving the crossover region of the other two strands for enhanced cleavage. At higher protein concentrations when DrHU forms a second complex, it binds similarly to the outer arms of strands 1 and 4 protecting the inner side of the two arms and in doing so it leaves the crossover region of strands 2 and 3 for enhanced cleavage [29]. However, with the removal of the N-terminus from DrHU, ΔDrHU forms a ladder of complexes. Densitometric profiles from two dimensional MPE-Fe(II) footprinting shows ΔDrHU binding the four-way junction DNA in the two fastest migrating complexes (complex 1 and 2), with protection of strands 2 and 3 in the crossover region. The binding mode seen for ΔDrHU is unique with the binding changing with each conformational isomer of 4WJ DNA. It is partly consistent with that observed for *E. coli* HU where it protects strands B and D of 4WJ DNA asymmetrically [10, 12] and forms a single DNA kink of ~65° at the break or junction due to intercalation of Pro63 of the β-arm while the α-arm binds the 3'-end, unable to kink the duplex DNA [13]. Thus the presence of the N-terminus of DrHU prevents the protein from binding to the crossover region of the 4WJ. The increased preference of linker histones for four-way junction DNA, which implies their role in DNA recombination, is consistent with a role for DrHU in DNA recombination [49].

While DrHU binds to the outer arms of the junction leaving the crossover region for enhanced cleavage, ΔDrHU binds asymmetrically to the junction crossover (Figure 3.5). Thus the N-terminus pulls the protein away from the crossover region to allow positioning of another

protein involved in DNA recombination. The interaction of DrHU with another protein upon binding the four-way junction DNA is also suggested by our EMSA studies. The complex formed with the cell lysate in presence of four-way junction DNA migrates differently than the DrHU-DNA complex. Upon incubation of the cell lysate with increasing amounts of DrHU there is formation of a stronger complex (Figure 3.7). This leads us to postulate that DrHU interacts with this unknown protein in the presence of the four-way junction DNA, stabilizing the complex.

The high resistance of *D. radiodurans* to extreme doses of ionizing radiation and prolonged periods of dessication may be a result of accurate and extensive template-independent DNA recombination events facilitated by restricted diffusion of DNA fragments, as well as template-dependent recombination [26]. It is conceivable that RecA protein, which stabilizes recombination intermediates by bringing together overlapping DNA fragments [27-28], is aided by DrHU due to its ability to stabilize four-way junction DNA structures on account of its unique placement.

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CHAPTER 4

SUBSTRATE SPECIFICITY OF *HELICOBACTER PYLORI* HU IS DETERMINED BY INSUFFICIENT STABILIZATION OF DNA FLEXURE POINTS

Introduction

Helicobacter pylori infection is associated with gastritis, peptic ulcer disease or gastric cancer [1]. *H. pylori*, which colonizes the human gastrointestinal tract, shows significant genetic diversity, reflected in sequence variations within otherwise well-conserved genes and by the presence of non-conserved genes, mobile genetic elements and chromosomal rearrangements [2-4]. Specialized to live in a single environment, *H. pylori* has a small genome (1.67 megabases), encoding a minimal set of metabolic genes, including specialized factors required for colonization and survival [5-7].

Bacterial genomes are compacted by association with histone-like proteins in a complex termed bacterial chromatin [8]. Most thoroughly characterized in *Escherichia coli*, the proteins primarily associated with the DNA are H-NS, Fis, HU and the HU homolog Integration Host Factor (IHF), all of which are present at concentrations up to or even exceeding 10 μM [9]. HU homologs are ubiquitous, but proteins with homology to *E. coli* Fis or H-NS are absent in many eubacteria, including *H. pylori*. HU may therefore be primarily responsible for genomic compaction and for specific regulatory functions in such organisms. Consistent with this notion, inactivation of the HU genes in *Bacillus subtilis* and in *Pseudomonas putida* was shown to be lethal [10, 11].

E. coli HU binds nonspecifically and with μM affinity to ~9 bp sites in duplex DNA, and with >100-fold higher affinity to cruciform DNA, specific DNA structures induced by

supercoiling, and DNA with nicks and gaps [12-16]. Although it is known that *E. coli* HU acts as an accessory factor in many processes, such as regulation of DNA supercoiling, recombination and repair [17,18], the molecular mechanisms whereby it executes these functions remain incompletely understood. HU proteins are dimeric, usually composed of 90-99 amino acid subunits forming a compact core of intertwined monomers from which two β-strands extend to embrace a DNA helix [19-21]. The structures of *E. coli* IHF or *Anabaena* HU in complex with DNA show that highly conserved prolines at the tips of these β-strands mediate two sharp DNA kinks. In the IHF-DNA structure, an ~160° DNA bend is generated by the two prolines intercalating between specific DNA base pairs, whereas *Anabaena* HU introduces a range of bend angles [22, 23]. Binding properties of HU homologs vary, with binding sites of 9-37 bp and affinities for duplex DNA between 5 nM and 2.5 μM [14, 24-29].

H. pylori experiences changes in the internal milieu that include transient pH fluctuations [4, 6, 7]. Predicted to play essential roles in regulation of nucleoprotein complex formation, *H. pylori* HU (HpyHU) would be expected to tolerate such changing conditions. Curiously, HpyHU was also shown to be among 13 proteins that are released from *H. pylori* by mechanisms other than nonspecific lysis and presumed to contribute to gastric inflammation and epithelial damage [30]. We show here that HpyHU is folded under pH conditions encountered *in vivo* and exhibits greater thermal stability compared to orthologs from other mesophiles. Whereas HpyHU shares certain properties with *E. coli* HU, such as the ability to introduce negative DNA supercoils, its DNA substrate specificity is distinct: it engages a longer DNA duplex, and it does not bind with significant preference to DNA with nicks, gaps and mismatches, but only to four-way DNA junctions. Our data suggest that the proline-mediated DNA bends must be stabilized by adjacent

residues, and that insufficient stabilization correlates with reduced bending and a failure to bind preferably to DNA with flexure points such as gaps and mismatches.

Materials and Methods

The gene encoding HpyHU was cloned from *H. pylori* genomic DNA (reference strain NCTC 11637), transformed into *E. coli* strain BL21(DE3)pLysS and the protein overexpressed as described [31]. HpyHU-RN was generated from the plasmid harboring the wild type gene as described [31]. Both proteins were purified as described [31, 32].

Circular Dichroism Spectroscopy

Circular Dichroism (CD) spectra were recorded on an AVIV Model 202 CD spectrometer. For wavelength scans, the protein concentration was 0.05 mg/ml in 10 mM sodium phosphate buffer, pH 7.0 with 50 mM NaCl. For thermal denaturation, the protein concentration was 0.1 mg/ml in the same buffer, and spectra were recorded from 190 to 250 nm in 1 nm steps at each temperature. Data were collected from 5°C to 90°C in 1.5-5° intervals, with the smallest temperature steps (1.5°) in the transition region. Protein was incubated for 6 minutes with stirring at each temperature in a 1 cm path length rectangular cuvette with a screw top seal. Reversibility of denaturation was measured to ensure that the system was at thermodynamic equilibrium, with the fraction of native protein recovered calculated from the CD values by linearly extrapolating the pre-transition and post-transition baselines. CD signals at 218, 219, 220, and 221 nm were used for analysis of the thermal denaturation curves which were fit to a modified form of the van't Hoff equation that simultaneously fits the native and denatured baselines and the transition region to obtain the Tm and ΔH° values for denaturation [33]:

$$\Delta\theta = [(m_n*T + b_n) + (m_d*T + b_d)]* K/(1 + K) \quad \text{and} \quad K = \exp((-\Delta H°(1 - T/Tm))/ RT)$$

where $\Delta\theta$ is the ellipticity, m_n and b_n are the slope and intercept of the native state baseline, m_d and b_d are the slope and intercept of the denatured state baseline, T is the temperature, Tm is the melting temperature, $\Delta H°$ is the van't Hoff enthalpy, and R is the gas constant. Tm and $\Delta H°$ values are reported as the mean of fits to 4 different wavelengths as a function of temperature. Data were fitted using the program KaleidaGraph.

Electrophoretic Mobility Shift Assay and Quantitation of Protein-DNA Complexes

Oligonucleotides used for preparation of 80 bp duplex or 37 bp duplex or duplex with loops, bulge-loops, nicks or gaps and oligonucleotides used for generation of four-way junction DNA were purchased and purified by denaturing polyacrylamide gel electrophoresis. To generate nicked DNA, two oligonucleotides (3′-GGATCCGATGTGGATGAG-5′ and 3′-AAACATTCTTAATTCGAAG-5′, terminating with free hydroxyl groups) were annealed to the 37-nt top strand. To generate DNA with a 1-nt gap, one nucleotide was omitted from the 3′-end of the second complementary strand while two nucleotides were omitted to generate DNA with a 2-nt gap. Integrity of the nicked/gapped DNA constructs was readily confirmed, as incompletely annealed constructs had distinct electrophoretic mobilities under the conditions used. DNA with two bulge-loops was generated by annealing the 37-nt top strand to 3′-GGATCCGATGTGGA**CCC**TGAGAAACA**CCC**TTCTTAATTCGAAG-5′, in which bulged-out nucleotides are shown in bold. The sequence of oligonucleotides used to generate four-way junctions were as reported [29]. DNA was ^{32}P-labeled at the 5'-end with T4 polynucleotide kinase, and equimolar amounts of complementary oligonucleotides were mixed, heated to 90°C and slowly cooled to 4°C to form duplex DNA. The 4-way junction was prepared by annealing strands 1-4, followed by purification of the junctions on native polyacrylamide gels. Electrophoretic mobility shift assays were performed using 10% (w/v) polyacrylamide gels (39:1

acrylamide:bisacrylamide) in TBE (45 mM Tris-borate (pH 8.3), 1 mM EDTA). Gels were prerun for 30 min at 20 mA at room temperature before loading the samples with the power on, except for experiments with bulged, nicked, gapped or four-way junction DNA for which electrophoresis was performed at 4ºC. DNA and protein were mixed in Binding Buffer, and each sample contained 50-100 fmol DNA in a total reaction volume of 10 µl, unless specified otherwise. After electrophoresis, gels were dried and protein-DNA complexes were visualized and quantified by phosphorimaging, using software supplied by the manufacturer (ImageQuant 1.1). The region on the gel between complex and free DNA was considered as complex. Complex dissociation during electrophoresis was measured as described [32], and the observed fraction of complex F(t) was corrected for dissociation during electrophoresis according to the equation $F_{corr} = F(t)/\exp(-k_{diss}*t)$, where k_{diss} is the exponential decay constant for complex dissociation during electrophoresis and t is time of electrophoresis.

Data were fitted to $y = y_{max} * [P]/([P]_{1/2} + [P])$ where [P] is the total protein concentration, $[P]_{1/2}$ is the protein concentration at half-maximal saturation, and y_{max} corresponds to maximal saturation.

DNA Cyclization Assays

Plasmid pET5a was digested with *Bsp*HI to yield a 105 bp fragment, which was ^{32}P-labeled at the 5'-end with T4 polynucleotide kinase. Ligation experiments with increasing concentrations of HpyHU were performed. Reactions were initiated by addition of 80 U of T4 DNA ligase to a final volume of 10 µl. Reactions containing 100 fmol DNA and the desired concentration of HU were incubated in 1X binding buffer (20 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, 0.1mM Na_2EDTA, 0.1 mM DTT, 0.05% (w/v) Brij58, 100 µg/ml BSA) with 1X ligase buffer (New England Biolabs) and 0.5 mM ATP at room temperature for 60 min

and terminated with 5 μl of 75 mM EDTA, 3 mg/ml proteinase K, 15% glycerol, and bromophenol blue and xylene cyanol, followed by a 15 min incubation at 55°C. Cyclized DNA was identified by its resistance to digestion by Exonuclease III. Reactions were analyzed on 8% polyacrylamide gels (39:1 (w/w) acrylamide: bisacrylamide) in TBE. After electrophoresis, gels were dried and ligation products visualized and quantified by phosphorimaging.

Results

Sequence Characteristics of HpyHU

The gene encoding the *H. pylori* HU homolog, HpyHU, was amplified from NCTC 11637 genomic DNA. Comparison with the sequence of HU genes from *H. pylori* strains J99 and 26695 reveals 18 positions of nucleotide polymorphism that translates into only four positions of amino acid variation (Figure 4.1). These variable amino acid residues are all found at positions where no overall consensus is present, as defined by alignment of 60 HU homologs [27]. This level of variation is equivalent to the overall level of strain-specific genetic diversity reported based on comparison of the J99 and 26695 complete genomic sequences [34].

In contrast, a comparison of the HpyHU amino acid sequences with the consensus sequence reveals ten positions at which HpyHU sequences differ; at all these positions, the three HU orthologs feature the same divergent residue, signifying a potential adaptation of HpyHU thermodynamic or DNA-binding properties to the unique needs of this organism (Figure 4.1). Overall similarity of structure is predicted by the conservation of hydrophobic residues in the protein core, and conservation of the DNA-intercalating Pro64 predicts DNA bending by the same mechanism as described for IHF and *Anabaena* HU [22, 23]. The conservative substitution of Phe for Leu in position 6, where helix-1 packs against helix-2 in the body of the protein, may not affect structure or DNA binding significantly. This substitution is also found in the sequence

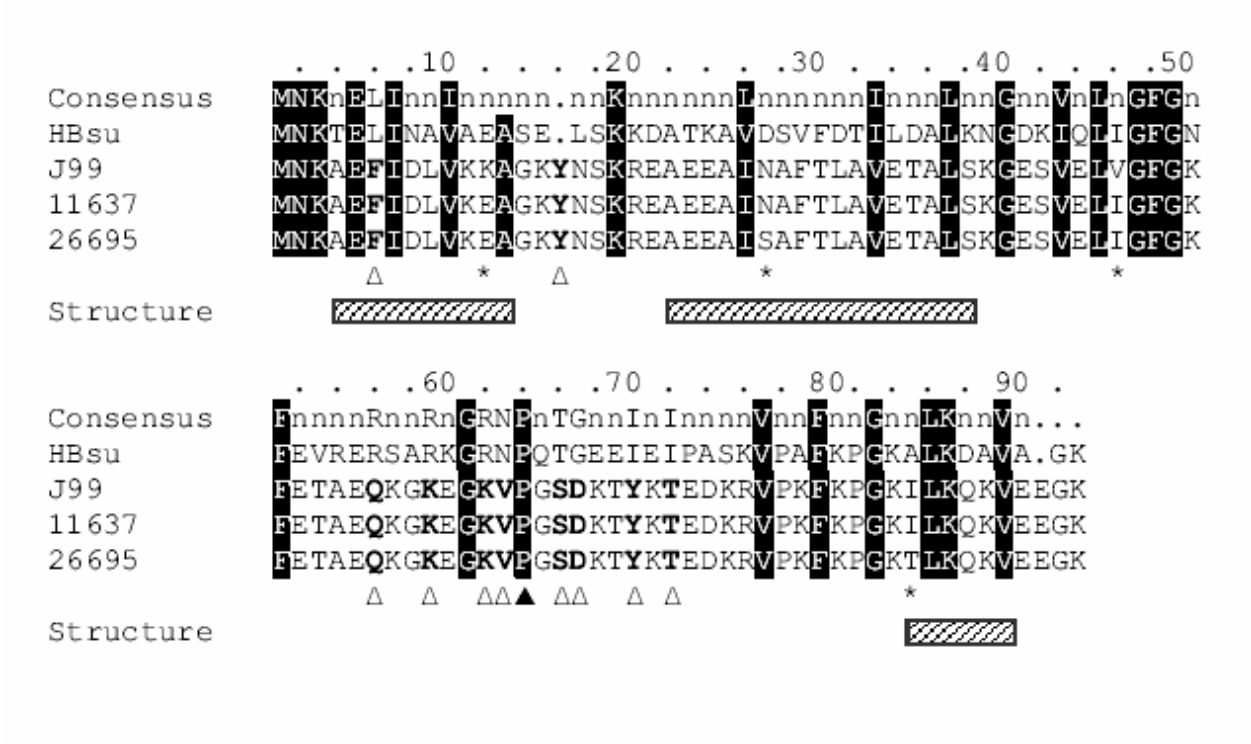

Figure **4.1**. Amino acid sequence alignment of HU from *H. pylori* J99, 11637 and 26695. The consensus sequence, derived from alignment of 60 HU homologs (with positions of >80% homology identified; [27]) is shown at the top, followed by the sequence of the *B. subtilis*-encoded HU homolog, HBsu. Residues are numbered based on the *H. pylori* HU sequences. Residues that vary between *H. pylori* HU sequences are identified with an asterisk. Residues that correspond to the overall consensus sequence are highlighted in black, and HpyHU-specific divergent residues are identified with an open triangle. The DNA-intercalating Pro64 is identified by the filled triangle. Helical segments, based on the structure of *B. stearothermophilus* HU, are indicated by hatched boxes below the alignment.

of HU from the closely related pathogen, *Campylobacter jejuni* [35, 36]. In position 16, a Tyr is inserted in the loop between helices 1 and 2. Increasing the length of this connecting loop may increase its flexibility and thereby optimize helix packing. Other HU homologs have amino acid insertions in this loop, however, it is absent from *C. jejuni* HU.

Eight substitutions are found between positions 56 and 72, in the β-ribbon arms that engage the DNA duplex (Figure 4.1). Except for Val63, none of these substitutions are found in *C. jejuni* HU. Gln56 corresponds to a position where replacement of a conserved Arg with Gln significantly impairs DNA binding by *B. stearothermophilus* HU [37]. Substitution of several residues at the tip of the DNA binding arms, surrounding the DNA-intercalating Pro64, is likely to generate distinct structural and dynamic properties and hence affect DNA binding.

Purification and Characterization of HpyHU and HpyHU-RN

The wild type HpyHU and the HpyHU-RN mutant protein in which Lys62 and Val63 were replaced with Arg and Asn, respectively; were purified as described [31]. These residues were chosen as the initial target for mutagenesis as Arg-Asn are almost completely conserved among HU homologs [27]. Our original expectation was, therefore, for the HpyHU-RN mutant protein to exhibit properties akin to those described for other homologs, with wild-type HpyHU possibly exhibiting distinct DNA-binding characteristics. As shown below, this expectation turned out not to be fulfilled.

The secondary structure of HpyHU was examined by CD spectroscopy (Figure 4.2a). Consistent with secondary structure predictions, significant α-helicity is evident. Spectra obtained in 10 mM sodium acetate pH 5.2 with 50 mM NaCl show that the protein remains folded in the acidic medium. Thermal denaturation was determined at pH 7.0 by recording the ellipticity at 218-221 nm at increasing temperatures, showing a gradual disruption of secondary

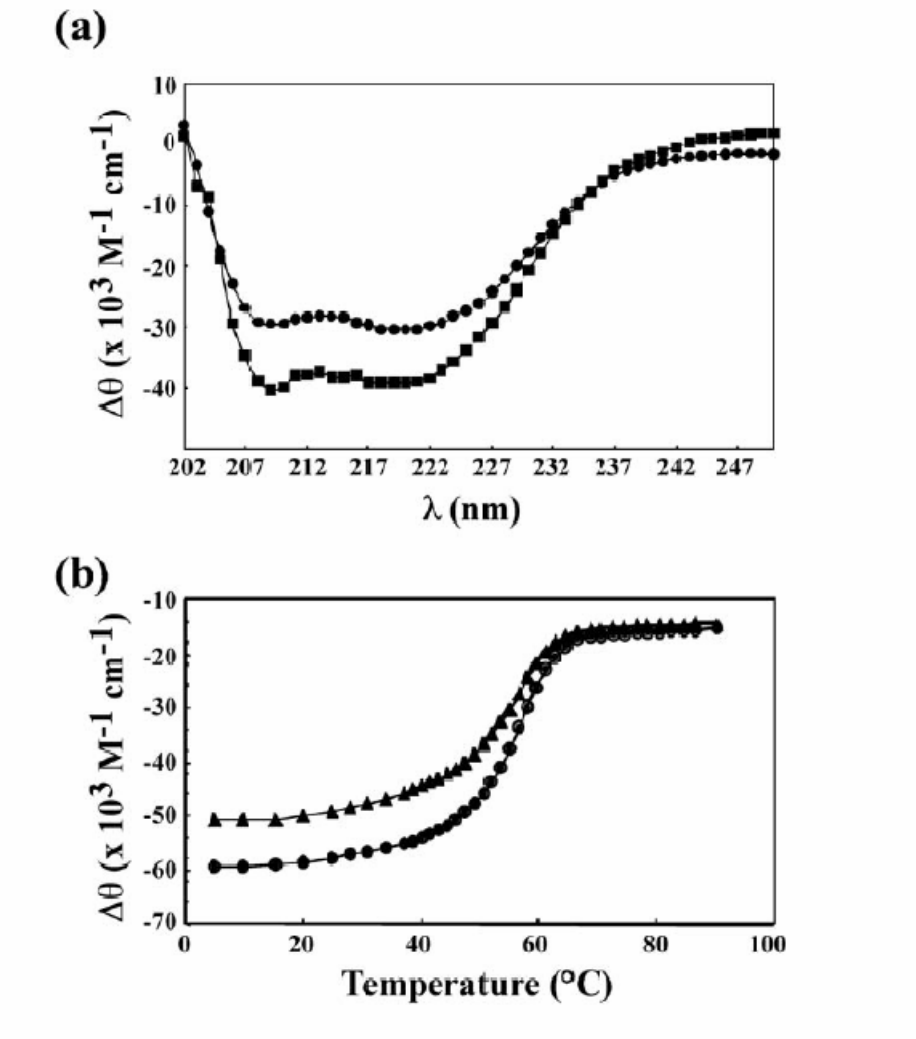

Figure **4.2.** Thermal stability of HpyHU. **(a)** Circular dichroism spectrum of HpyHU at room temperature, recorded in phosphate buffer pH 7.0 (●) or in acetate pH 5.2 (■) **(b)** The ellipticity as a function of temperature. The midpoint of the temperature transition corresponds to the melting temperature Tm. Denaturation curve (●), renaturation curve (▲).

structure (Figure 4.2b). HpyHU exhibited 85% reversible denaturation with two successive denaturations yielding a melting temperature Tm of 56.4 ± 0.1ºC and a van't Hoff enthalpy change at Tm of 55.6 kcalmol^{-1}. Evidently, HpyHU is more thermally stable than *B. subtilis* HBsu (Tm = 48.6ºC in sodium phosphate buffer pH 7.0 containing 200 mM NaCl; [38]).

Affinity for Duplex DNA

In analogy with HU from *E. coli* and *B. subtilis*, HpyHU is expected to bind DNA nonspecifically, implying the existence of numerous overlapping sites on the DNA. The modified version of the McGhee-von Hippel binding isotherm for nonspecific binding to a finite DNA lattice was used to evaluate the data [31]. Although it is theoretically possible to determine the nonspecific site size, n, the observed equilibrium association constant, Ka, and the cooperativity parameter, ω, simultaneously from a 3-parameter fit, experimental uncertainty may render such a fit ambiguous. Therefore, the occluded site size was first determined by stoichiometric titrations (where [DNA]>Kd) using a 37 bp duplex and 80 bp duplex DNA. The binding site size of 20 ± 1 bp was measured at 50 mM KCl on 37 bp DNA and 19 ± 1 bp on 80 bp DNA [31] which is larger than the site sizes of non-specific binding for *E. coli* HU (~9 bp, measured by counting complexes on 21-42 bp duplexes or by fluorescence anisotropy and analytical ultracentrifugation using 13 or 34 bp DNA; [12, 39]) or IHF (9-16 bp, depending on salt concentration, measured by Isothermal Titration Calorimetry using 14 bp and 160 bp DNA; [40]), but equivalent to the suggested site size for *Anabaena* HU (19 bp, estimated from crystallographic data; [23]). Binding affinity was measured on 148 bp duplex DNA; while fits to the non-cooperative McGhee-von Hippel equation failed to converge (R = 0.6875), fits to the cooperative McGhee-von Hippel equation showed modest cooperativity of binding (ω = 64 ± 6)

and Kd = 2.1 ± 0.5 μM (R = 0.9947) [31]. This value of Kd is comparable to the 2.5 μM affinity reported for *E. coli* HU at 200 mM NaCl [14].

HpyHU Bends DNA

To assess the ability of HpyHU to bend DNA, ligase-mediated cyclization assays were performed. This assay measures the efficiency with which T4 DNA ligase mediates ring closure of DNA fragments that are shorter than the persistence length. As shown in Figure 4.3, HpyHU mediates cyclization of a 105 bp DNA fragment, although not nearly as effectively as HBsu (lane 2). The HpyHU-RN mutant protein does not mediate DNA cyclization above that seen in absence of HU protein, consistent with its reduced affinity and very limited ability to supercoil DNA [31]. While DNA bending by HpyHU is expected based on properties of other HU proteins, the failure to bend DNA characteristic of HpyHU-RN suggests that residues surrounding the DNA-intercalating proline are important for stabilizing the DNA kinks.

HpyHU Binds Preferentially to Four-way Junction DNA

Binding to 37 bp perfect duplex DNA was compared to loop-containing DNA in which two 4-nt loops are placed in the DNA, symmetrically disposed about the center and with a spacing of 9 bp [31]; this DNA construct was shown to serve as a preferred substrate for other HU homologs, including *Thermotoga maritima* HU (which has an ~37 bp site size; [27]) and *Anabaena* HU (~19 bp site size; [25]). The midpoint of the binding isotherm for HpyHU binding to perfect duplex DNA (35 ± 3 nM) is only ~3-fold higher than for binding to looped DNA (12 ± 1 nM; indicating that HpyHU binds DNA with increased flexure with only a modest preference [31]. The modestly increased affinity for looped DNA is also seen with 37 bp DNA containing two bulge-loops separated by 9 bp of duplex (Figure 4.4). Bulge-loops, which confer predisposed DNA bends, evidently do not provide optimal complementarity with the HpyHU binding

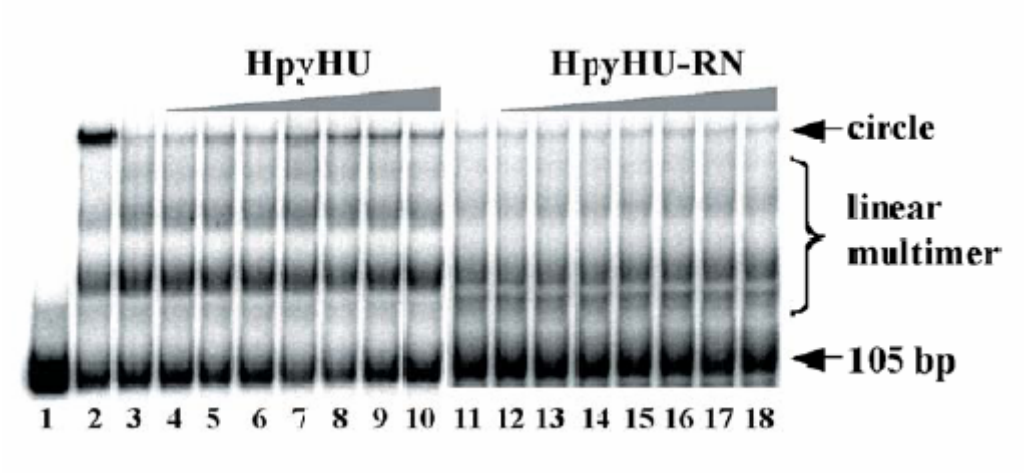

Figure **4.3**. HpyHU bends DNA. 105 bp DNA was incubated with T4 DNA ligase for 60 min in the presence of 2 pmol HBsu (lane 2) or 0-7 pmol HpyHU, respectively (lanes 3-10). Reaction in lane 1 contained no ligase. Less than 10% of total DNA is cyclized at the highest [HpyHU]. HpyHU-RN (1-7 pmol) does not cyclize the 105 bp DNA (lanes 12-18) above that seen in absence of HU (lane 11). DNA was resolved on an 8% polyacrylamide gel.

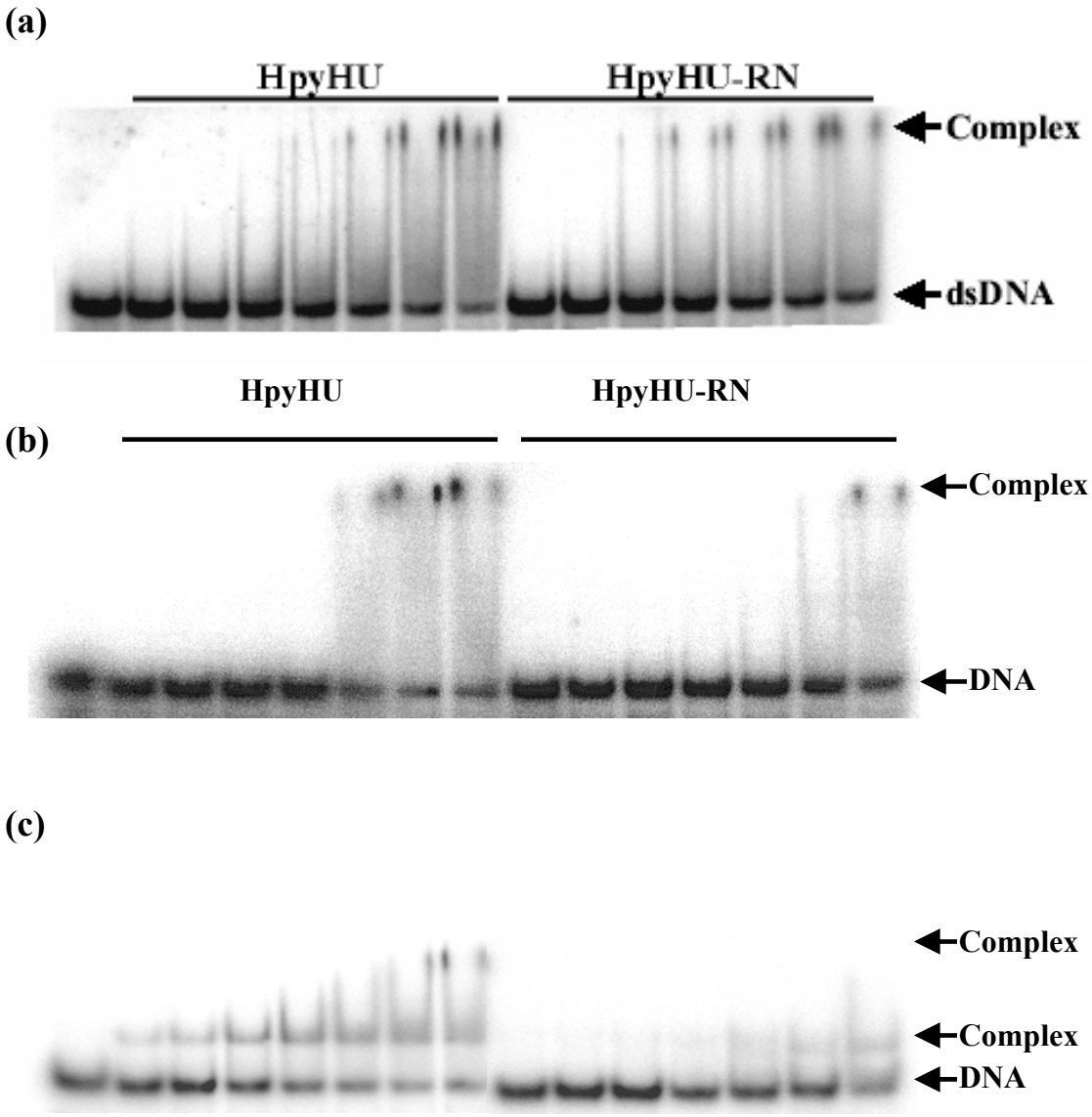

Figure **4.4**. Electrophoretic mobility shift assay of HpyHU binding to more flexible DNA. Titration of HpyHU (left panel) and HpyHU-RN (right panel) with (a) 37 bp duplex DNA (b) 37 bp duplex DNA with 3′end overhang, and (c) 37 bp DNA with bulges. Complex and free DNA is indicated on the right. HpyHU variants are identified at the top, and protein concentrations (identical for both panels) are as follows: Lane 1, no protein, Lanes 2-8 and 9-15, reactions with 5, 10, 25, 50, 100, 250, 500 nM, protein respectively.

interface to yield stable complex formation. Introduction of a central nick or a 1-2 nt gap (terminating with free hydroxyl groups) into the 37 bp duplex does not result in enhanced complex formation compared to perfect duplex, nor does the generation of a 3'-overhang, again suggesting a limited ability of HpyHU to engage DNA with greater flexibility stably (Figure 4.4).

HpyHU-RN which binds the short duplex DNA comparably to wild-type HpyHU (half-maximal saturation of 50 ± 6 nM [31]), does not bind markedly better to the looped DNA compared to perfect duplex, indicating that increased flexure at potential sites of kinking fails to stabilize complex formation significantly. The almost complete lack of preference for distorted DNA is consistent with the limited ability of HpyHU-RN to bend and supercoil DNA [31]. Complex formation measured at pH 6.0 is equivalent to that seen at pH 8.0 [31].

Four-way junction DNA was generated based on the sequence used for analysis of *E. coli* HU [12]. Two stable HpyHU complexes, whose high mobility suggests a compacted structure, are formed at low protein concentration, and an additional complex is seen at higher concentrations (Figure 4.5). No evidence of cooperativity of binding was observed. This pattern of complexes is similar to that observed with *E. coli* HU, where two HU dimers bind to opposite angles of the four-way junction, leading to two complexes at low protein concentrations, with higher-order complexes corresponding to HU binding to the linear branches [12]. HpyHU binds the four-way junction with half-maximal saturation of 5.3 ± 0.5 nM. HpyHU-RN also binds preferentially to the four-way junction, the higher half-maximal saturation of 13.7 ± 2.2 nM reflecting the already observed reduced binding affinity of the mutant protein (not shown). The significant preference for the pre-bent DNA construct suggests that only DNA in which the

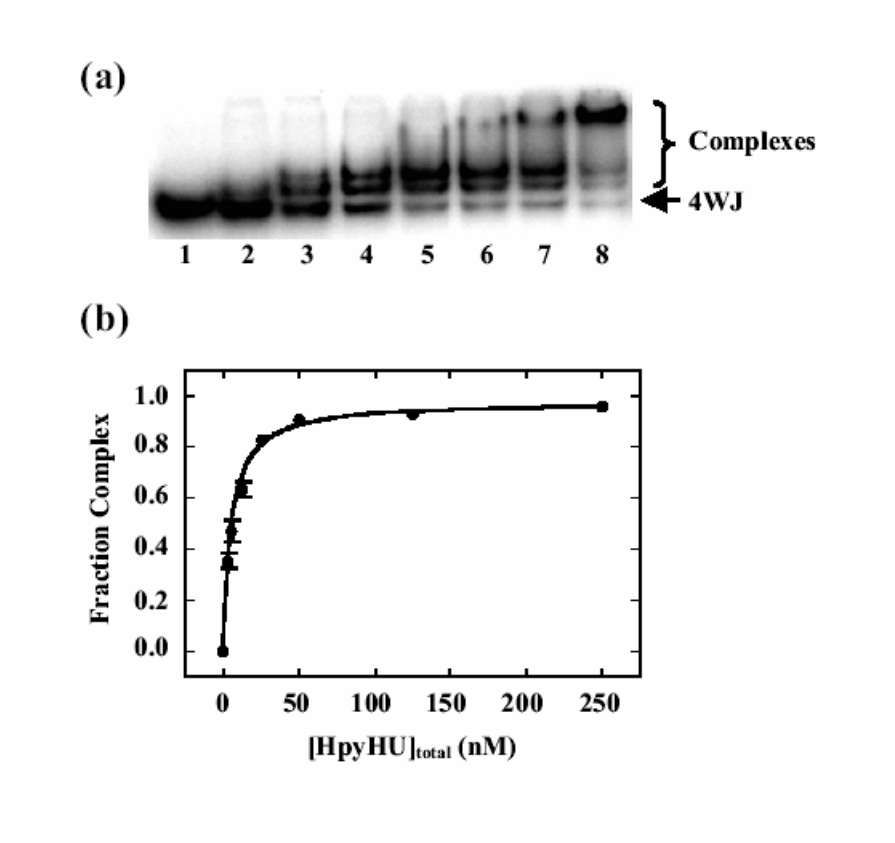

Figure **4.5.** HpyHU binds preferentially to four-way junction DNA. Titration of HpyHU with four-way junction DNA. Binding was assayed in the stacked X conformation preferred in the presence of $MgCl_2$ [53]. Complex and free DNA is identified at the right. Protein concentrations are 0, 2.5, 5, 12.5, 25, 50, 125, and 250 nM, respectively.

energetic cost of bending has been significantly lessened serves as an optimal substrate for HpyHU.

Discussion

Thermal Stability of HpyHU

Comparison of HpyHU sequences from three strains reveals several amino acids that differ from the overall consensus (Figure 4.1). One of these substitutions is an insertion in the loop connecting helices 1 and 2, and this connecting loop also features a Gly in position 14. It was previously shown that loop flexibility correlates with optimal helix packing and thermal stability: substituting Gly15 with Glu in *B. stearothermophilus* HU reduces the loop flexibility and causes a reduction in Tm from 64ºC to 54ºC, and increasing the loop flexibility through replacement of Glu15 with Gly in *B. subtilis* HU (HBsu) or in the *B. subtilis* bacteriophage SPO1-encoded HU homolog, TF1, in both cases increases the Tm by 11ºC [38, 41, 42]. In general, Gly15 is found in HU encoded by thermophilic organisms, such as *B. stearothermophilus* and *Thermus aquaticus,* and insertions in this connecting loop are found for instance in HU from *Aquifex aeolicus* [27]. Kawamura and co-workers (1996) reported a Tm for HBsu of 48.6ºC, measured by CD spectroscopy in phosphate buffer pH 7.0 with 200 mM NaCl, a Tm that is significantly lower than the 56.4ºC measured for HpyHU under somewhat less stringent conditions (phosphate buffer pH 7.0 with 50 mM NaCl; Figure 4.3). Additional reports have been published presenting a Tm for HBsu (33°C, 39.7ºC and 43ºC, respectively [43-45], based on CD spectra recorded in sodium cacodylate pH 7.5 with 100 mM KF (Tm = 33°C) or under unspecified solution conditions). Based on the correlation between flexibility of the loop connecting helices 1 and 2 and thermal stability, optimized packing mediated by flexibility of this connecting loop is likely the basis for the enhanced stability of HpyHU [38, 41, 42]. We note

that the CD spectrum at pH 5.2 shows that HpyHU remains folded at lower pH (Figure 4.2); under these conditions, HBsu was shown to be partially unfolded and the unfolded monomer unable to dimerize [46]. It may be that the enhanced thermal stability is an accidental consequence of evolutionary pressures to remain folded when exposed to transient increases in intracellular acidity.

Four-way Junction DNA is an Optimal Substrate for HpyHU

HpyHU binds nonspecifically and with low affinity to an ~19 bp DNA site, and its preference for DNA with greater than average flexure is modest [31]. This is in remarkable contrast to *E. coli* HU which was reported to bind ~9 bp of linear duplex DNA with at least 100-fold lower affinity compared to DNA with nicks and gaps (Kd = 2-8 nM in 200 mM KCl; [13]). Loops or discontinuities in the DNA backbone are considered to increase local flexure, thereby facilitating formation of the protein-mediated DNA kinks [47-49]. However, HpyHU has only modest preference for such flexible sites [31], suggesting an inability to stabilize the proline-mediated DNA kinks significantly, even when DNA flexure is greater than average. The biological corollary may be that HpyHU *in vivo* does not discriminate significantly between perfect duplex DNA and DNA with breaks or mismatches, arguing against a role in recognition of substrates for the DNA repair systems. In contrast, HpyHU has significant binding preference for DNA in which the energetic cost of bending has been reduced by pre-bending the DNA (Figure 4.5). Such properties would be consistent with *in vivo* roles in stabilization of four-way junction structures or other severely bent DNA conformations. HpyHU introduces negative DNA supercoiling [31], consistent with a role in compaction of the bacterial genome.

Substrate pecificity of HU Proteins is Determined by their Ability to Stabilize DNA Bends

Upon substitution of Lys-Val preceding the DNA-intercalating proline with Arg-Asn, DNA binding and bending is attenuated. Notably, preference for DNA with flexure points such as gaps or mismatches is also lost, while binding to prebent DNA is only modestly reduced. It is conceivable that the HpyHU-specific substitutions within the DNA-binding β-arms render a conformation that is incompatible with a spatial disposition of Arg that permits DNA-contacts, and that Lys is specifically required for electrostatic contacts to the DNA. The significant effect of the KV→RN substitutions on DNA bending and on preference for DNA with imposed flexure suggests that a stabilization of the proline-mediated DNA kinks by adjacent residues is essential. We propose that insufficient stabilization of the DNA kinks leads to a failure to bend duplex DNA and an inability to utilize flexible DNA as a preferred substrate, while retaining a significant preference for pre-bent DNA, as seen for HpyHU-RN. Such properties were recently shown also to characterize the *Deinococcus radiodurans*-encoded HU homolog, which binds preferably only to four-way junction DNA and which fails to bend DNA [29]. A modest stabilization of the flexible DNA hinges would correlate with preferred binding to more flexible DNA sites, as seen for *E. coli* and *Anabaena* HU [12-14, 23, 32]. The distribution of charged residues in the region surrounding the DNA kink has been previously shown to have a marked effect on affinity and substrate specificity; for example, variants of the SPO1-encoded HU homolog TF1 with Ser or Gln in the position corresponding to Arg62 of the consensus sequence fail to bind DNA unless it is pre-bent [50]. Accordingly, substrate specificity of HU proteins appears to vary as a function of the ability of individual proteins to stabilize a severely bent DNA conformation.

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CHAPTER 5

SUMMARY AND CONCLUSION

HU proteins have drawn attention for their nature of interaction with DNA and pleiotrophic role in eubacteria. First characterized in *E. coli* as a histone-like protein for introducing negative supercoils into relaxed DNA molecules in the presence of topoisomerase I [1], it has more recently been shown to play a role in DNA repair [2, 3], DNA recombination and DNA replication [4]. *D. radiodurans*, a soil dwelling mesophile, has a highly proficient system for repair of double-strand breaks formed upon exposure to extreme doses of γ- and UV-irradiation and dessication [5]. Till date, scientists are exploring the possible ways through which this extreme capacity of survival is maintained. To better understand the puzzle about the repair mechanism of this unique radiation resistant organism, we initiated studies with *D. radiodurans* HU focusing on its possible contribution.

Sequence and Structural Considerations

DrHU has a unique 47 amino acid extension at the N-terminus, followed by the DNA-binding fold characteristic of type II DNA-binding proteins (Figure 2.1). The DNA-intercalating Pro at position 63 at the tip of the β-arms is conserved, but the surrounding residues are divergent in the *Deinococcus* and *Thermus* groups, with Arg at position 61 replaced by Val, and Asn at position 62 replaced by Arg or Lys [6]. In *H. pylori* HU, this GRNP motif is replaced by GKVP, and restoring the GNRP motif in the mutant protein leads to significant changes in DNA binding [7] indicating that amino acid substitutions at the tips of the DNA-binding β-arms significantly affect substrate specificity. The presence of Gly at position 15, in the loop connecting helices 1 and 2, is noteworthy as it is otherwise associated with HU from thermophiles (e.g. *B.*

stearothermophilus and *Thermotoga*) where it is known to contribute to loop flexibility with optimal helix packing and increased thermostability [8-11].

Both DrHU and ΔDrHU form dimers upon cross-linking with gluraldehyde (Figure 2.2 and 3.1) which suggests that the dimer interface is essentially unaltered in the full-length and truncated proteins, compared to other HU homologs [8-10]. Based on the far-UV spectrum of ΔDrHU (Figure 3.2), the secondary structure has a polypeptide conformation similar to HU homologs [9, 11], which together with the cross-linking results suggests that the N-terminus with a predicted kinked helix conformation exists as a separate domain of DrHU.

The 47 amino acid extension at the N-terminus of DrHU has three APA(A/K)K repeats, reminiscent of the (S/T)PKK repeats found in the C-terminus of histone H1. The C-terminal tail is disordered in aqueous solution, and adopts a helical conformation on DNA binding or in trifluoroethanol [12], where the charged lysines bind DNA through charge neutralization and facilitate DNA compaction [12, 13]. Due to the similarity of the repeat sequences, the N-terminus of DrHU is probably also disordered in aqueous solution due to charge repulsion and participates in DNA binding.

Differential Substrate Selectivity

Differential substrate selectivity by HU homologs is evidenced, for example, by the variable length of binding sites engaged by members of HU family. While *E. coli* HU is reported to bind only ~9 bp of duplex DNA [14], *H. pylori* HU and *Anabaena* HU require ~19 bp duplex DNA [7, 15] and IHF, TF1 and *T. maritima* HU are shown to bind to ~37 bp sites [6, 16, 17]. These proteins share the ability to bend duplex DNA; IHF, TF1 and *E. coli* HU has distinct preference for distorted DNA such as loops and nicks [16-18]; and *T. maritima* HU mediates DNA circularization and supercoiling *in vitro* (unpublished results). DrHU has a binding site size

of ~50 bp duplex DNA (Figure 2.5) which has been reduced to ~17 bp for ΔDrHU (Figure 3.4), suggesting that the increased binding site is due to the stable positioning of the N-terminal tail on the DNA. DrHU is unique in its inability to bend duplex DNA as evidenced by the failure of both the full-length and truncated proteins to induce DNA circularization (Figure 2.8 and data not shown). It is the first member of the type II DNA binding proteins whose binding characteristics argue against its role as an architectural protein of mediating local DNA distortions. However, the marginal preference of both DrHU and ΔDrHU for DNA with loops and nicks (Figure 2.4b and 3.3b) or hmU-for-T substitutions is due to the presence of both torsional and bending flexibility.

While DrHU has modest ability to introduce supercoils into relaxed plasmid DNA (Figure 2.7), ΔDrHU is unable to form any supercoils (Figure 3.5), which is consistent with the interpretation that the N-terminal tail of DrHU contacts DNA and with the contribution of the lysine rich repeats in DNA condensation [12]. As ΔDrHU interacts with DNA directly it may be that the path of wrapping does not allow the formation of supercoils.

Analysis of the DNA binding properties of HU from another mesophile, *H. pylori*, (HpyHU), indicates that HpyHU has a modest preference for looped (12±1 nM) or bulged DNA over perfect duplex DNA (35±3 nM). Like DrHU, it has no distinct preference for flexible DNA with nicks, gaps, or overhangs as indicated with *E. coli* HU [19]. It is capable of bending DNA by forming circles in linear duplex DNA (Figure 4.3), and it introduces supercoils into relaxed plasmid DNA. With a distinct preference for four way junction DNA, half-maximal saturation of 5.3 ± 0.5 nM (Figure 4.5), HpyHU reveals a significant preference for pre-bent DNA where the energetic cost of bending is lessened.

Thermal Stability

Although DrHU has Gly at position 15 as seen in HU from thermophiles, it does not conserve the GFG motif at the dimer interface, but the F is substituted for L which may affect the hydrophobic core of the protein and as a consequence the thermal stability. This is observed in circular dichroism studies of ΔDrHU where the secondary structure conformation is in agreement with that of other HU proteins [11], but the Tm of 46.4˚C is similar to that of *B. subtilis* HU, HBsu, and not to that of *B. stearothermophilus* HU. Whether the absence of the N-terminal tail or the cold-sensitive behavior of ΔDrHU is responsible for this Tm require further studies. In *H. pylori* HU, an insertion in the loop connecting helices 1 and 2 and Gly at position 14 is likely to confer increased loop flexibility and thermostability as observed with a Tm of 56.4˚C (Figure 4.2) compared to HBsu which has a significant lower Tm of 48.6˚C [20]. HpyHU has been suggested to have enhanced thermal stability to withstand transient exposure to intracellular acidity based on the observation that it remains folded at pH 5.2, whereas HBsu, is shown to be only partially folded under the same conditions. The lack of enhanced thermal stability for ΔDrHU is also consistent with the reported thermal stabilities of two unrelated *D. radiodurans*-encoded proteins (a hypothetical uricase regulator (HucR) [21] and starvation inducible DNA-binding protein (personal communication)), neither of which exhibits enhanced thermal stability.

Substrate Specificity

DrHU and ΔDrHU exhibit marked preference for four-way junction DNA, as observed also for the *E. coli* HU homolog [22, 23]. This DNA exists as an equilibrium mixture of two conformational isomers with an interduplex angle of 40-60˚ between the stacked junction arms in the presence of Mg^{2+} [24, 25]. DrHU forms two complexes on binding four-way junction DNA, and its position distal to the crossover to the inner arms of strands 2 and 3 (complex 1) alternates

with its position on the inner side of the outer arms of strands 1 and 4 (complex 2), exposing the junction to enhanced cleavage (Figure 2.6). This positioning of DrHU disfavors protein-protein contact as evidenced by the lack of cooperativity. ΔDrHU forms a ladder of complexes, and the position of the two fastest migrating complexes show that the protein protects the crossover region by protection of strands 2 and 3 (Figure 3.6). For *E. coli* HU, the two protein molecules bind by the β-arms preferentially to the crossover region of strands B and D with the α-helical bodies positioned away from the junction such that such that the two molecules have no protein-protein interaction, separated by a minimum distance of ~6Å [23].

The position of DrHU on the outer arms of the four-way junction DNA suggests that it may stabilize the junction while allowing another protein to access the junction crossover. The direct interaction of DrHU with another protein from the cell lysate in the presence of four-way junction DNA by forming a stronger complex (Figure 3.7) suggests that it is involved in DNA recombination. As DrHU is expected to be present in significant amounts (μM) in the cell [26] with a high affinity for the four-way junction DNA, it may recognize these recombination intermediates formed as a consequence of DNA damage caused by exposure to environmental extremes in *D. radiodurans* and direct other recombination specific proteins (the concentration of which is now increased [27, 28]) to the junction region in the repair of the double-strand fragments. A functional RecA protein is required for bringing together overlapping DNA fragments in *D. radiodurans* resistance phenotypes [27, 29], and other recombination proteins include Rec Q helicase [30] and the junction resolving enzymes Ruv A, B or C [31]. The latter may be speculated to interact with DrHU as it serves to stabilize four-way junction structures in preparation for subsequent repair events.

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VITA

Sharmistha Ghosh was born in Durgapur, India, and attended school in Carmel Convent, Durgapur. She was then awarded the Honors Bachelor's degree with a 1st class in zoology. She then moved on to the master's program and graduated with a 1st class in zoology with a special paper in Genetics and Molecular Biology. From 1997-1999 she worked as a Junior Research Fellow in Chittaranjan National Cancer Institute, Calcutta, where she had experience working with anticarcinogenic reagents in regard to skin cancer. She enrolled in the doctoral program in the Department of Biological Sciences at Louisiana State University in the fall of 2000. There, she studied the DNA binding properties of the histone like protein HU from a radiation resistant mesophile, *Deinococcus radiodurans* and its probable role in DNA recombination processes under the guidance of Dr. Anne Grove. As a graduate student she taught introductory biology and advanced biochemistry lab courses. She will complete the requirements for the Doctor of Philosophy degree in biochemistry in December 2004. In January 2005 she plans to move to Boston, Massachusetts, and join Dr. Charles Richardson in the Department of Biological Chemistry at Harvard Medical School as a postdoctoral research fellow.